# Extraction of Antioxidant Compounds from Olive (*Olea europaea*) Leaf

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# Abstract

Olive leaves are by products of olive oil industry, which are also regarded as a rich source of antioxidants. The objective of the present work was to extract antioxidant compounds from olive leaves. The effects of extraction conditions on the total phenolic content were investigated. Three extraction methods were used in this research for recovery of phenolic compounds from olive leaves. A multilevel experimental design was implemented with the aim of optimising the recovery of phenolic compounds from olive leaves by using nontoxic water/ethanol-based solvent. The factors considered were (i) the extraction time, (ii) the extraction temperature, (iii) solvent: solid ratio and (iv) the ethanol concentration. The results suggest that a good recovery of phenolic compound from olive leaves may be achieved at 40°C with a solvent/ solid ratio of 30:1 and ethanol concentration of 80 % (v/v). Drying of fresh leaves before extraction is highly recommended to achieve better recovery of important phenolic compounds including oleuropein. Ultrasonic probe may be useful to improve extraction efficiency and also reduce extraction time. The quantitative and qualitative determinations of phenolic compounds were performed by high-performance liquid chromatography (HPLC), which revealed that oleuropein, luteolin-4-O-glucoside, luteolin-7-O-glucoside and apigenin 7-O-glucoside were the major phenolic compounds present. In this study phenolic compounds extracted from olive leaves of two cultivars (Frantoio & Barnea) were analysed. A comparison among two cultivars shows quantitative differences in some phenolic compounds. The antioxidant capacities of the extracts were evaluated by measuring the radical scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical and by using Oxygen Radical Absorbance Capacity (ORAC). Olive leaf extracts exhibited high antioxidant capacity which suggests olive leaf extract is effective in the function of scavenging free radical. The stability of olive leaf extract stored at four temperatures has also been investigated. The results show increasing temperatures caused greater extent of degradation of phenolic compounds. The best storage condition for olive leaf extracts was at -20 °C in absence of light and oxygen.

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# Chapter One: Introduction

It is well known that free radicals generated in vivo can cause oxidative stress, consequently causing damage to DNA, proteins and lipids in biological systems. This damage has been associated with human disease such as atherosclerosis, rheumatoid arthritis and inflammatory bowel disease. Antioxidants can significantly delay or prevent oxidation of targeted substrate by scavenging free radicals (Halliwell et al., 1995; Sanchez-Moreno, 2002).

In a food system, the oxidative deterioration of lipids in foods is responsible for rancid odours and flavours, with a consequent decrease in nutritional quality and safety. It is a major concern of the food industry. The addition of antioxidants will sufficiently protect lipids from oxidation and effectively preserve flavour and colour (Moure et al., 2001).

Fruits and vegetables and related products such as juice and wine are major sources of natural antioxidants in the diet. Other sources of natural antioxidant are herbs, spices, teas where a large diversity of phenolic compounds have been found in those foods (Yanishlieva-Maslarova & Heinonen, 2001). For the food industry, synthetic antioxidants are very effective, inexpensive and stable; however, they have certain disadvantages, including possible toxicological effects (Kiritsakis et al., 2010). Therefore recovery and utilisation of antioxidants from natural sources have gained increasing attention, in particular from plant origin (Moure et al., 2001). By-products or wastes from plant food processing are a very promising source of antioxidants from both economic and ecological points of views.

Olive tree (*Olea europaea*) belongs to the family of Oleaceae, which is cultivated for its edible fruits. Olive fruits are consumed as table olives and used for producing olive oil. The traditional `Mediterranean diet' is considered to be one of the healthiest. In this diet, olive oil is the most popular dietary lipid. The leaves of the olive tree have also been widely used in folk medicine in regions around the Mediterranean (Soler-Rivas, 2000).

Olive leaves are by-products from olive tree cultivation and olive oil mills. Considerable amount of olive leaves are principally generated during pruning of trees and harvesting and working of olives. In general, olive leaves are composted in original farms or burned, industrial use of olive leaves is initially limited to animal feeds (De Leonardis et al., 2008).

Recent research shows that antioxidant compounds derived from leaves and fruits of olive trees are excellent antioxidants. Olive leaf extracts have been strongly associated with health and preservation. The health effects of olive oil and olive leaves are mainly due to the presence of bioactive compounds (Mourtzinos, 2007). Oleuropein, the most abundant bioactive compounds in olive leaves can prevent cardiac disease by protecting membrane lipid oxidation, acting on coronary dilation, improve lipid metabolism and protect enzymes and hypertensive cell death in cancer patients (Japon-Lujan et al., 2006a). The addition of olive leaf extract into edible oils (olive oil, sunflower oil, palm oil) can substantially improve antioxidant capacity and oxidative stability of the oil (Andrikopoulos et al., 2007).

Olive trees are found predominantly in the Mediterranean region and are also increasingly developing as a commercial crop in South America, New Zealand and Australia. The olive industry in New Zealand has developed extensively since 1998.Wairarapa and Hawkes Bay are two of the most important regions in the North Island for the production of extra virgin olive oil with several very large commercial groves well established. Nelson and Marlborough are the two largest and most important olive growing regions in the South Island of New Zealand (Edwards, 2006b). Utilisations of olive leaves to produce health promoting products and food ingredients have great potential for both the nutraceutical and food industry within New Zealand.

There are many methods for the extraction of antioxidants from olive leaves, and solvent extraction is the most common one. The solvents frequently used for the extraction of oleuropein and derivatives from olive leaves are methanol and water mixtures or hexane. However, the toxicity of both solvents limits their industrial exploitation in order to obtain antioxidants for human use undesirable (Japon-Lujan et al., 2006a). Thus, the main objective of this project was to extract antioxidants from olive leaves using nontoxic and cheap solvent and specifically to

1) Determine the best extraction method amongst three proposed methods
2) Determine the optimum extraction conditions, under which the good recovery of antioxidants from olive leaf can be obtained
3) Identify and determine major phenolic compounds present in olive leaf extracts
4) Determine antioxidant capacity of olive leaf extracts
5) Investigate the effect of storage conditions on the stability of olive leaf extracts

# Chapter Two: Literature Review

The literature review in this section consists of three sections: firstly, a brief overview on olives and their potential benefits on human health, New Zealand olive industry, and the herbal supplements market and related regulation, secondly, followed by an in depth review of the scientific techniques which may apply in extraction and evaluation of phenolic compounds from olive leaf, and lastly a brief review of industrial processes for obtaining an olive leaf extract.

## 2.0 Introduction

Olive (*Olea europaea*, family Oleaceae) is an evergreen tree grown for its edible fruits, which yield oil and are also marketed as table or pickled olives. The olive has been closely associated with human religious, sociocultural, medicinal, and nutritional needs (Raina, 2003). The olive tree (*Olea europaea*) is one of the most important fruit trees in the world; almost 98% of all olive trees are planted in Mediterranean countries (Bouaziz & Sayadi, 2005). Spain, Italy, and Greece are the leading producers of olives (Raina, 2003).

The olive tree is amongst the oldest known cultivated trees in the world. It is uncertain the exact origin of the olive tree, the genetic and archaeological studies indicated that the original centres of olive cultivation were Palestine, Lebanon, Syria, Cyprus and Crete. The olive tree was firstly wide spread on the Greek islands and the mainland of Greece, Italy, and then probably introduced into Spain by the Greeks, Romans and Arabs (Kiritsakis, 1998). The Romans invented the press used to take the oil from olive fruits. The olive tree was widely cultivated in Southern Europe and this is why it is called *Olea europaea.* The expansion of olive tree has continued from Mediterranean countries to California, South Africa, Australia, Japan, China, Indian and other countries. The earliest record of olive oil production in California was in 1803 and the first trees were planted in Sydney, Australia around 1805. Today, the olive tree is cultivated all over the world (Kiritsakis, 1998).

The New Zealand olive history can be dated back to 1835, when Charles Darwin visited the northern-most region of the country and reported olive trees growing in those areas (Edwards, 2006b). However, olive trees were not planted in commercial groves until the 1990's, and the New Zealand Olive Association (later renamed as Olives New Zealand) was formed few years later to undertake research and to provide networking opportunities for the olive industry. The olive industry has developed extensively since 1998 when a

tasting panel for virgin olive oil was established. Nowadays there are approximately one million olive trees planted in commercial groves, principally for the production of extra virgin olive oil. In 2006, there were 1600 tonnes of olives for producing approximately 190,000 litres of extra virgin olive oil (Edwards, 2006b). Commercial olive groves in New Zealand are planted with a relatively narrow range of cultivars, namely: 'Frantoio', 'Leccino', 'Pendolino', 'Moraiolo', 'Koroneiki', 'Manzanilla', 'Picual', 'Barnea', 'Chemlali', 'Nebali', 'Picholene' and 'Verdale'. Amongst all these cultivars, the Italian cultivars are most common in commercial groves but there are also significant plantings of 'Barnea', 'Manzanilla', 'Koroneiki' and 'Picual'. Wairarapa and Hawkes Bay are two of the most important regions in the North Island for the production of extra virgin olive oil with several very large commercial groves well established. Nelson and Marlborough are the two largest and most important olive growing regions in the South Island of New Zealand. The harvest begins in Northland around mid-April. The central growing regions usually begin their harvest in late May but in the southern-most area the olives will not be harvested until mid to late June (Edwards, 2006a).

## 2.1 Olives and Human Health

### 2.1.1 Harvest of Olives

Traditionally, the olives are harvested at the green-yellow or black-purple stage. Harvest techniques include harvest after natural fall, from tree by the hand or beating the branches or with a tree shaker. Hand picking from the tree undoubtedly yields the fruits of the best quality for both table olive and olive oil, however, the labour expense is considerably high. Olives can also be collected by beating the branches of the tree with long poles so that the fruit falls on to cloth sheets lying on the ground after the fruit have turned black. Mechanical shakers with stationary cloth, canvas-covered, or mechanized collecting frames are used to a limited extent, mostly for harvesting oil olives (Kiritsakis, 1998; Raina, 2003).

Olive fruits are consumed as table olives, or used for extraction of olive oil. The major commercial table olives include Spanish style green fermented olives, American style canned ripe olives and Greek style black (naturally ripe) olives. Turkey is one of the world's biggest producers and consumers of table olives. Olive oil was the most important foodstuff in the Mediterranean diet. Olive oil has been classified into four groups according to the method of preparation and acid content: virgin olive oil, refined olive oil,

blended olive oil and industrial oils (Raina, 2003). Besides being used as a food, olive oil was used in pharmaceutical products, as well as for body care, lighting and religious ceremonies. The main by-products of oil processing are olive pomace, waste water, and the olive leaves. There is a significant amount of phenolic acids present in olive pomace and waste water such as gallic acid and caffeic acid. The pomace has been used for further extraction of olive pomace oil (Kiritsakis, 1998). The olive leaves have been used as animal feeds, but it was also extensively exploited as a source of antioxidants for applications such as cosmetic, therapeutic and food industries (Boudhrioua et al., 2009).

### 2.1.2 Chemical Composition of Olives and Olive Oil

The average chemical composition of the olive fruits and leaves are shown as Table 2.1. Water accounts for half of the olive fruits and leaves weight. Water serves as a solvent for water soluble constituents of both fruits and leaves. Carbohydrates in olive fruit mainly include sugar and cellulose. A decrease in sugar content of olive fruit with maturation is related to an increase of oil contents (Kiritsakis, 1998; Boudhrioua et al., 2009). Minor components of various minerals such as iron, potassium, manganese, magnesium and copper are found in both olive fruits and leaves (Kiritsakis, 1998; Fernandez-Escobar et al., 1999).

Table 2.1: The Average Chemical Composition of Olive Fruits and Leaves (Kiritsakis, 1998; Boudhrioua et al., 2009; Fernandez-Escobar et al., 1999).

| **Composition (g/100g fresh weight)** | **Moisture** | **Protein** | **Lipid** | **Ash (minerals)** | **Carbohydrates** |
|---|---|---|---|---|---|
| **Olive fruit** | 50.0 | 1.6 | 22.0 | 1.5 | 24.9 |
| **Olive leaf** | 49.8 | 7.6 | 1.1 | 4.5 | 37.1 |

Lipid or oil is the most important constituent of olive fruits; it is composed of triacylglycerols and small quantities of free fatty acid, glycerol, pigments, flavour compounds, sterols, etc. The fatty acids present as glycerides in “Cornicabra” virgin olive oil are shown as Table 2.2 (Salvador & Giuseppe, 2010). The olive oil is rich in oleic acid, a monounsaturated fatty acid and it has been associated with beneficial physiological effects (Kiritsakis, 1998).

Table 2.2: Total Fatty Acid Composition (%) of Olive Oil (Salvador & Giuseppe, 2010)

| **Fatty acids** | **Range** |
|---|---|
| Palmitic (C16:0) | 6.99-11.05 |
| Plamitoleic acid(C16:1) | 0.49-1.11 |
| Stearic acid (C18:0) | 2.61-4.43 |
| Oleic (C18:1) | 76.52-82.49 |
| Linoleic(C18:2) | 3.07-6.62 |
| Linolenic acid (C18:3) | 0.48-0.95 |

### 2.1.3 Phenolic Compounds in Olives and Olive Oil

Other important constituents of olives are various phenolic compounds. The major phenolic compounds present in olive fruits are anthocyanins (cyanidin and delphinidin glucosides), flavonols (mainly quercetin-3-rutinoside), flavones (luteolin and apigenin glucosides), phenolic acids (hydroxybenzoic, hydroxycinnamic, others), phenolic alcohols (tyrosol and hydroxytyrosol), secoiridoids (oleuropein, demethyloleuropein, ligstroside, nuzhenide), and verbascoside, a hydroxycinnamic acid derivative (Boskou, 2006a).

The phenolic compounds in olive oil may be phenolic acids, simple phenols like tyrosol and hydroxytyrosol, secoiridoid derivatives of the glycosides oleuropein and ligstroside, lignans and flavonoids. A wide range of total phenols (50 – 1000 mg/kg) have been reported in olive oils. Polar phenols and tocopherols are important for the stability of virgin olive oil. It also attributes to the bitterness and pungency of olive oil (Boskou, 2006a).

Table olives have a phenol composition that differs from that of olive oil and non-processed olives. This is due to the debittering process, which causes diffusion of phenols from the fruit to the water or brine. The prevailing phenols in table olives are hydroxytyrosol, tyrosol, luteolin, and phenolic acids. Higher levels ranging from 100 – 760 mg/kg were found in Greek-style black olives and Spanish-style green olives in brine. Hydroxytyrosol and caffeic acid are eliminated during the preparation of California-type black olives (Boskou, 2006a). The composition and levels of phenolic compounds in olive leaves will be discussed in detail later in this review.

### 2.1.4 Potential Beneficial Effect on Human Health

Olive oil is extracted from the fruit. This is the main purpose of the olive tree grown worldwide. It is well known that the beneficial effect of olive oil is attributed to its favourable fatty acid profile and minor components such as carotenoids, phospholipids and phenolic compounds (Soler-Rivas et al., 2000). The research in the recent decade indicates that the phenolic compounds of olive oil and olive leaves, in particular, oleuropein have been associated with a reduced incidence of hypertension, cardiovascular diseases, diabetes and hyperlipidemia due to its antioxidant activity and its antihypertensive, anti-inflammatory, hypoglycaemic and hypocholesterolemic properties. The function of oleuropein in vivo may include protecting human cell membrane from lipid oxidation, affecting coronary blood vessel dilation, improving lipid metabolism and preventing hypertensive cell death in cancer patients. However, there is lack of information on the bioavailability of these olive polyphenols (El & Karakaya, 2009). Recent research showed olive leaf extracts containing polyphenols such as oleuropein and hydroxytyrosol could reverse the chronic inflammation and oxidative stress that induce cardiovascular, hepatic, and metabolic symptoms in a rat model of diet-induced obesity and diabetes without changing blood pressure (Poudyal et al., 2010).

Traditionally, olive tree leaves have been used as a folk remedy for fevers and other diseases such as malaria (Benavente-Garcia, et al., 2000). The antimicrobial activity of olive polyphenols has also been widely investigated by scientists, and in-vitro tests discovered that oleuropein and its hydrolysis products could inhibit the growth of a broad spectrum of pathogenic micro-organisms, including bacteria (*Staphylococcus aureus ,Salmonella enteritis, Bacillus cereus, Escherichia coli, Bacillus cereus T spores*), fungi, viruses and parasitic protozoan, but have no effect against yeast. The precise mechanism of antimicrobial action remains uncertain, but it appears that oleuropein could interfere with the synthesis of amino acids necessary for the survival of micro-organisms. Nevertheless, in-vivo studies are still required to confirm whether this broad antimicrobial activity of oleuropein can occur in the human body (Soler-Rivas et al., 2000; Lee & Lee, 2010). Due to promising health benefits of olive fruits and leaves, the “Mediterranean diet” which is rich in olive oil is considered to be one of the healthiest diets, and olive leaf extract is also suggested to have great potential as a food ingredient, dietary supplement and source of natural antioxidants (Benavente-Garcia et al., 2000; Lee & Lee, 2010).

## 2.2 Herbal Supplement Market and Related Regulations

### 2.2.1 Herbal Supplement Market

The New Zealand supplement market has been growing 5% annually as reported in 2008 (Gruenwald, 2008). The users are mainly female, of 'NZ European and others' descent, well-educated and relatively young (Smith, Wilson & Parnell, 2005). Consumers' interests in natural products and preventative health are likely to drive interest in dietary supplements. By comparison, the sales of herbal and traditional products in Australia have increased by 27.5% since 2004 and reached sales of $551 million in 2009. The herbal dietary supplement sectors account for 29.5% of total health product market value, with 0.2% of sales attributed to olive leaf extract manufactured by Olive Products Australia Pty Ltd (OPA), a company specialising in producing olive leaf extract from fresh-picked leaves (Euromonitor, 2009b). OPA was bought by Comvita New Zealand Ltd in 2008, an NZX-listed natural health company based in the Bay of Plenty and well known for its bee-derived health products. In the same year, Comvita launched its "Olive Leaf Complex" in New Zealand and worldwide, and this product won the 'Best New Product' category of the UK's 2008 Health Food Business (Comvita New Zealand, 2008; Olive Leaf Australia, 2010).

With regards to the global market, in 2006 the top three herbs featured globally in medicines, supplements and functional foods were ginseng, ginkgo and noni in 2006 (Gruenwald, 2008). Herbal and traditional dietary supplements accounted for 48% of the total health supplement retail sales in 2009, while child-specific products showed the fastest growth rate (Euromonitor, 2009a).

### 2.2.2 Regulations in Australia and New Zealand

In New Zealand, currently many natural health products in oral dose forms are regulated as dietary supplements under the Dietary Supplements Regulations 1985 (which sit under the Food Act 1981). Other natural health products are regulated as related products, medicines or herbal remedies under the Medicines Act 1981(Ministry of Health, 2010). Under Dietary Supplements Regulation 1985, "dietary supplement" means an amino acid, edible substance, herb, mineral, synthetic nutrient, or vitamin, alternatively, it is intended to supplement the amount of above nutrients normally derived from food. When the products are sold as dietary supplements, therapeutic claims of these products are prohibited under current Dietary Supplements Regulations 1985 (Beattie David, 1985). However, it is likely

to be covered under the joint Australia New Zealand healthcare and therapeutic products legislation in the future (New Zealand Food Safety Authority, 2010).

Under Current "*Australian Therapeutic Goods Regulations 1990*" (Australia Government Department of Health and Ageing Therapeutic Goods Administration, 2010)

"Herbal Substance" means all or part of a plant or substance (other than a pure chemical or a substance of bacterial origin): (a) that is obtained only by drying, crushing, distilling, extracting, expressing, comminuting, mixing with inert diluents or another herbal substance or mixing with water, ethanol, glycerol or aqueous ethanol; and (b) that is not subjected to any other treatment or process other than a treatment that is necessary for its presentation in a pharmaceutical form.

According to the consultation paper "Regulation of Herbal Substances in a Joint Australia New Zealand Therapeutic Products Agency" (Australia Government Department of Health and Ageing Therapeutic Goods Administration, 2005)

All ingredients entered into the joint Agency therapeutic products database will need to be expressed using approved names. In the case of herbal substances, most ingredients will require three parts to the approved name – the botanical name, the plant part name and the plant preparation name. Where a dry, fresh or powdered herb has been further processed, sponsors in Australia are currently required to include, in the expression, solvent and extraction details (where relevant), and the equivalent dry or fresh weight of the original herbal material used. For example, a 500 ml olive leaf extract (natural flavour) made by Olive Leaf Australia is expressed as follows:

"Each 5mL contains *Olea europaea* (Olive) leaf extract equiv. to approx. (5g) 5,000mg fresh leaf, standardized to contain 22.0mg oleuropein"

A 50 capsules "Oliviral" olive leaf extract made from dried olive leaf by Nutra-Life Health & Fitness (NZ) Ltd is expressed as follows:
"Each capsule contains Herbal Extracts Dry Conc. Equiv. Dry *Olea europaea* (Olive) leaf 1.46g, Equiv. Oleuropein 48.67mg". The standardised Olive Leaf Extract used in OLIVIRAL capsule is standardised using HPLC analysis technique.

Despite widespread use, there is currently no regulatory definition for the term 'Standardization'. Although the term is meant to refer to the quantity of the herb from which the preparation was derived. Generally, a standardised herbal substance is an herbal preparation (usually an extract) made to a consistent and specified level of an identified component, or group of components. The primary aim of 'standardisation' is to assist in delivering a specified level of one or more phytochemicals derived from the original herbal starting material (David, 2004).

### 2.2.3 Olive Leaf Extracts in New Zealand Market and Its Main Ingredients

Major olive leaf extract products selling in the New Zealand Market are shown in Table 2.3, there are mainly in two forms, liquids and powders and most of products are powders encapsulated in a capsule. The level of oleuropein varies amongst different products, and powdered extract is generally higher than that of liquid extract with a range from 30mg to 110mg per capsule. For liquid olive leaf extract, each 5ml extract contains 16mg to 32mg oleuropein depending on the different brands.

The highest levels of oleuropein are claimed in Good Health "Viralex Liquid Olive Leaf Extract" for a liquid product and NFS "Olive Leaf Ultra" for a powdered product respectively. Comvita olive leaf complex is the only product which claims to be extracted from fresh olive leaf, and it contains 22mg oleuropein per 5ml in a vegetable glycerine base. Other main ingredients in the olive leaf extract in general are Astragalus Root, dried yeast, Pau d Arco and zinc amino acid chelate. Astragalus root is herbal medicine known as "huang ji" in China, which is one of the most popular herbs used for increasing stamina and endurance, and improving resistance to the cold. Pau d Arco is a traditional herb native to South America and famous for its remarkable health benefits, it can be used as remedy for inflammations and infectious (Chevallier, 2000). Dried yeast mainly -"Saccharomyces cerevisiae"- is used in the product as a source of beta-1-3 glucan, and zinc amino acid chelate as a source of zinc. Liquid extracts are normally in a base of honey, glycerine, ginger, vanilla tincture, ethanol and apple juice concentrates.

Table 2.3: Common Olive Leaf Extract Products in the NZ Market and Their Oleuropein Levels

| Brands | Product Name | Product form and its main Ingredients |
|---|---|---|
| **NFS** | Olive Leaf Ultra | ***Each capsule contains***: Olive Leaf Ext. equiv. to dry leaf 5,500mg Equiv to ***110mg Oleuropein.*** |
| | Vir-Attack | ***Each capsule contains***: Olive Leaf extract 2200mg, equiv to ***Oleuropein 44mg***, Garlic extract, Andrographis, Echinacea purpurea extract, and rhizome , Thyme extract, , Zinc citrate. |
| Nature's Way | Olive Leaf standardised | ***Each capsule contains***: Olive, dried leaf extract 250mg standardised to ***12% oleuropein (50mg)*** and Olive leaf 250mg, Gelatin (capsule), magnesium stearate, millet. |
| | Olive Leaf with Echinacea & Vit C | ***Each capsule contains***: Proprietary Blend 450mg (Olive leaf extract 12%, ***90 mg oleuropein***, Echinacea purpurea), Vitamin C 60mg |
| **Goodhealth** | ViraleX children | ***Per 5ml***. Herbal extracts equivalent to dry: Olea europaea Leaf (Olive) 540mg, equiv. ***Oleuropein 16mg***, Astragalus Root, Pau d Arco. Other Ingredients: Saccharomyces cerevisiae (Dried Yeast), providing Beta 1-3 Glucan, Zinc amino acid chelate. **In a base of New Zealand honey**, glycerine, ginger, vanilla tincture, ethanol and apple juice concentrates. |
| | ProOlive combines herbal extracts | ***Each Capsule*** Contains: Herbal Extracts Equivalent to Dry: Olea europaea (Olive) Leaf 1250mg equiv. ***oleuropein 30mg*** Herbal Extracts Equivalent to Fresh: Echinacea purpurea Root 200mg |
| | Viralex | ***Each capsule*** provides: Herbal extracts equivalent to Dry: Olive leaf 1.08 grams, equivalent ***Oleuropein 32mg***, Astragalus root extract, Pau D'arco bark extract, Saccharomyces cerevisiae (Dried Yeast), providing Beta 1-3 Glucan, Zinc amino acid chelate. |
| | Viralex Liquid | Ingredients ***per 5ml***: Herbal extracts equivalent to dry: Olive leaf 1.08 grams, Equivalent ***Oleuropein 32mg***, Astragalus root, Pau d Arco bark. Other Ingredients: Saccharomyces cerevisiae (Dried Yeast), providing Beta 1-3 Glucan, Zinc amino acid chelate. **In a base of apple juice concentrate**, glycerine, vanilla tincture and ethanol. |
| MicroGenics | Olive Leaf Extract | Ingredients ***per 5 ml*** Extract equiv. to dry: Olive (Olea europaea) leaf 5000mg equiv. ***Oleuropein 22mg*** |
| Greenridge | Liquid Olive Leaf Extract | Each 2ml contain: ***Olea europaea (Olive) extract equiv. to 1g (1000mg) dry leaf Ethanol*** |
| Comvita | Olive Leaf Complex | Each ***5ml*** herbal extracts equivalent to: ***Fresh Olea europaea Leaf: 5g*** (5000mg) standardised equiv. ***Oleuropein 22mg*** Sweetened with glycerine (vegetable) ,Free from alcohol, added sugar, yeast gluten, artificial colour flavours and preservatives |
| Nutra-Life | Oliviral | ***Each capsule*** contains: Herbal Extracts Dry Conc. Equiv. Dry Olea europaea (Olive) Leaf 1.46g Stand. Equiv. Oleuropein 48.67mg Astragalus membranaceus Root , Pau D'Arco, Stem Bark, Sambucus nigra (Elder) Flower, Zinc (as Gluconate) , Yeast Dried (a source of Beta 1-3. Glucan) |
| **Thompson's** | Organic Olive Leaf extract | ***Each capsule*** contains: Concentrated organic herbal extract equivalent to dry Olive (Olea europaea) leaf 5g (5000mg) providing ***not less than 100mg Oleuropein*** per capsule |
| Healtheries | Olive Leaf extract | ***Each capsule*** contains: Olive Leaf Extract 3500mg (standardised to provide ***Oleuropein 30mg***) |

All data is sourced from: Healthy (2011) and Healthpost (2011)
http://www.healthy.co.nz/news/198-olive-leaf-extract-super-immune-system-booster.html
http://www.healthpost.co.nz/

## 2.3 Extraction of Phenolic Compounds from Olive Leaves

### 2.3.1 Sample Preparation

Fresh olive leaf generally needs drying and milling before extraction. As a preservation method, drying is carried out to remove the water from the leaves consequently protecting the leaves against spoilage as well as some phenolic against degradation by enzyme action. Milling the dried leaves can reduce particle size and facilitate solvents entering into the cells of the leaves. It also improves extraction efficiency or extractability. Many different drying approaches have been explored by researchers, but air drying, microwave drying and freezing drying have been mostly reported in the literatures. Air drying can be carried out at room temperature or elevated temperatures for different time periods (Savournin et al., 2001; Bouaziz & Sayadi, 2005; Japon-Lujan & de Castro, 2006; Malik & Bradford, 2008). Generally longer times are required for lower temperatures to achieve the same extent of dryness of leaves. Careful temperature control is needed during the drying since it may cause degradation of polyphenols. Malik & Bradford (2008) reported that air drying at 25°C or elevated temperature such as 30 °C and 40°C in the oven results in good recovery of oleuropein, however, drying at 60°C would result in substantial losses of polyphenols possibly due to degradation of oleuropein and other polyphenols. Freezing drying is an alternative way to effectively avoid thermal degradation while removing the water from leaves. The leaves are immediately frozen in liquid nitrogen, and lyophilized before extraction (Briante et al., 2002; Goula et al., 2010). The elimination of water through lyophilization generally does not affect the phenolic compounds excessively, and allows samples to be kept for longer periods (Escribano-Bailon & Santos-Buelga, 2003). The temperature of -80 °C is commonly used to store biological samples; it is also used for storage of plant tissues for the purpose of keeping bioactive compounds in the plant. However it was not recommended as a sample preparation method for olive leaves extraction, thawing frozen olive leaf samples caused a sharp reduction in oleuropein levels. That is possibly due to breakage of cell membrane during thawing and consequently release of active oleuropein-degrading enzymes. Furthermore, small discrepancies in sample handling would easily cause inconsistent results when frozen and stored at -80°C. (Malik & Bradford, 2008)

Microwave drying was reported to avoid ester hydrolysis of saponins which occurs during air drying, and higher concentrations of oleuropein have been reported after using this

drying method (Savournin et al., 2001; Bouaziz & Sayadi, 2005). Infrared drying was recently suggested as a good method for preserving olive leaves because it allows the retention of the green colour of fresh leaves. Infrared drying resulted in considerable moisture removal from the fresh leaves (more than 85% by weight) during a short drying period (varying from about 162 min at 42°C to 15 min at 70°C). The total phenolic content of infrared dried olive leaves was greater compared to fresh ones (Boudhrioua et al., 2009).

There is little literature reporting extraction of phenolic from fresh olive leaves, and it was reported that increased levels of oleuropein were found in dried leaves than in fresh leaves probably due to the conversion of oleuropein glucoside into oleuropein by β-glucosidase present in fresh leaves (Silva et al., 2006).

### 2.3.2 The Methods of Extraction

Traditionally, there are a number of methods to make herbal remedies such as infusions, tinctures and decoctions, and many substances have been used as a base for extracts. Infusion is the simplest way to prepare leaves and flowers for use as a medicine. By simply pouring boiling water into a handful of dried herbs and infuse for a certain length of time, water based extracts are obtained (Chevallier, 2000). Tinctures are made by soaking dried or fresh herbs in alcohol for 10-14 days, and then removing the herbs using a wine press. Syrups are effective preservatives and can be combined with infusions and decoctions to make syrups, which have additional benefits for cough mixtures as well as relieving sore throats. With sweet taste, syrups can disguise the taste of unpalatable herbs and are generally appreciated by children. With capsule herbal remedies, gelatine or vegetarian capsules are often used (Chevallier, 2000).

Solvent extraction was the main method adopted by most researchers to extract phenolics from olive leaves. This is a process designed to separate soluble compounds by diffusion from a solid matrix using a liquid matrix. This process takes place by two steps, which are the adsorption of solvent into the solid phase by osmotic forces, by capillary and by solvation of the ions in the cells, then followed by diffusion from the solid phase (Escribano-Bailon & Santos-Buelga, 2003). The aim of extraction is to concentrate antioxidant components of raw materials; the extraction process involves a more or less vigorous agitation of the ground raw materials with extraction solvent at ambient or elevated temperatures and subsequent separation of the residue by filtration. Repeated

extraction steps may be accomplished to increase the extract yield. Alternatively, a packed bed of the ground material can be used which is leached by the extraction solvent under refluxing conditions (Oreopoulou, 2003).

Besides conventional solvent extraction, other methods such as ultrasound- assisted, microwave-assisted and supercritical fluid extraction can offer a good yield and preserve the properties of antioxidants. These methods can be used for the extraction of polyphenol from plant tissue. Ultrasound assisted extraction is faster and more complete in comparison with traditional methods (Escribano-Bailon & Santos-Buelga, 2003). The use of ultrasound can increase extraction yield and shorten extraction time, therefore it was suggested by Albu et al. (2004) that sonication appears to have great potential as a method for the extraction of antioxidants from Rosmarinus officinalis for the food and pharmaceutical industry (Albu et al., 2004). Ultrasound assisted extraction can be carried out by either mixing ground sample with the suitable solvent, and placing into the ultrasonic bath where the working temperature and extraction time are set or by using an ultrasonic probe (Adam et al., 2009).

Microwave assisted extraction is a new technique that combines microwave with traditional solvent extraction. Some studies shows that it has many advantages over conventional extraction methods include higher extraction rate, simple and cheap. It has been used for extracting phenolic compounds from tea leaves and grape seeds (Escribano-Bailon & Santos-Buelga, 2003).

Supercritical fluid extraction greatly facilitates the extraction process and reduces extraction time due to low viscosity and relatively high density of supercritical fluid; it can also minimize possible degradation because it can operate in absence of light and air. Supercritical carbon dioxide is the most widely used extraction solvent (Escribano-Bailon & Santos-Buelga, 2003).

In terms of olive leaf extraction, microwave and ultrasound assistance during extraction were proposed by Japon et al. (2006) to accelerate the extraction and reduce the extraction time. Complete extraction of targeted analytes can be achieved in 8 minutes and 25 minutes when using microwave and ultrasound assistance, respectively (Japon-Lujan et al., 2006a, 2006b). The high recovery and the low maintenance costs make it potential for industrial implementation. Le Floch et al. (1998) used carbon dioxide modified with 10% methanol as a supercritical fluid to isolate phenolics from olive leaves, however, extraction

did not achieve satisfactory yield. Too many variables such as modifier, temperature, pressure and collection system resulted in difficulty in controlling extraction (Le Floch et al., 1998). A static–dynamic superheated extraction approach for olive leaves was developed by Japon et al. (2006a) in order to obtain more concentrated extracts. This method involved high temperature and pressure, which may cause thermal degradation of phenolic compounds (Japon-Lujan & de Castro, 2006). A recent study provided a relatively simple and rapid method, the olive leaf powder was refluxed with 80% ethanol at 80°C for 3 hours, and the extraction was repeated three times (Lee et al., 2009). Another recent research used fresh olive leaves and ethanol and the extraction took place at room temperature for two weeks (Poudyal et al., 2010). After extraction, suspensions were filtered using filter paper or glass wool or centrifuged. The solvent was removed by various methods such as rotary evaporators, vacuum evaporators and freezer drier, and then a dry or a concentrated extract was obtained (Savournin et al., 2001; Bouaziz & Sayadi, 2005; Lee et al., 2009).

There are a number of important parameters which affect extraction yield of antioxidants. They are the type of solvent, concentration of solvent, particle size, extraction temperature and time, the ratio of liquid-to-solid and pH of solvent. Before extraction, pre-treatment may be needed for various purposes such as removal of lipid or chlorophyll. Purification of the crude extracts may be essential, especially for the alcoholic extracts, in order to remove the undesirable co-extracts and improve the antioxidant properties and to create a product with a light colour, odour and taste. Isolation of an antioxidant fraction rich in flavonoids from alcoholic extracts can be achieved through solvent evaporation under vacuum and subsequently liquid-liquid extraction by ethyl ether. An enriched flavonoid fraction can be obtained by repeated extraction of the aqueous layer with equal volume of ethyl acetate (Oreopoulou, 2003).

### 2.3.3 The Effect of The Extracting Solvent

Both the extraction yield and antioxidant capacity of extracts are strongly influenced by the solvent, due to the different polarity and different antioxidant potential of compounds extracted (Oreopoulou, 2003). Therefore, organic solvent of higher polarity are more effective in quantitative recovery of phenolic compounds than non-polar solvent and methanol was reported in many studies as a good solvent for extraction of phenolics from the plants including olive leaves. However, it may lead to unacceptable levels of toxic residues in the final extracts; ethanol and water are the most widely employed solvents for

safety and abundances reasons (Moure et al., 2001; Oreopoulou, 2003). Altiok et al.(2008) revealed that ethanol alone was not effective as a solvent for extraction of phenolic compounds from olive leaves, and water has important role in extraction process by increasing the diffusion of extractable polyphenols through the plant tissues (Altiok et al., 2008). Changes in ethanol concentration modify the physical properties of the solvent such as density, dynamic viscosity, and dielectric constant. Solubility of compounds would also be modified by changes in the ethanol concentration, and this may influence the extraction of phenolics (Cacace & Mazza 2003). Mylonaki et al. (2008) used 40%, 50% and 60% ethanol (v/v) in the study to investigate the effect of ethanol concentration on total phenolics yield. They discovered that the ethanol concentration in the medium had seemingly biphasic effect, as an intermediate ethanol level (50%) appeared to provide the lowest yield. In contrast, the trends observed when decreasing or increasing the ethanol concentration indicated that polyphenol extraction was favoured in both cases. Since many of the olive leaf phenolics are glycosides, it can be assumed that their solubility may be higher at 40% ethanol (v/v) than at 50% ethanol (v/v). On the other hand, oleuropein, which is significantly less polar, would be more soluble in 60% ethanol (v/v) than in 50% ethanol (v/v). This may be the reason for such a biphasic tendency in response to ethanol changes (Mylonaki et al., 2008).

Ethanol, methanol, ethyl acetate, boiling water, hexane, diethyl ether, chloroform and butanol were the main solvents used by researchers for olive leaf. Of these solvents, aqueous methanol or ethanol was most commonly used and the concentration of solvent varied between 40% and 80% (v/v). Extraction with 80% methanol (v/v) was reported as the most effective method for olive leaf polyphenols (Malik & Bradford, 2008). Boiling of dried leaves was also a very efficient method for extracting oleuropein and verbascoside resulting in 96 and 94% recoveries of these compounds, respectively (Malik & Bradford, 2008). Japon et al. (2006b) suggested 80% aqueous ethanol (v/v) was the optimum solvent for extraction of the targeted phenolics from olive leaf and it can be used as replacement for toxic solvents (methanol, diethyl ether, chloroform ) to obtain bioactive phenols for human use (Japon-Lujan et al.,2006b). Lee et al. (2009) also reported total flavonoid and phenolic contents were significantly higher in the 80% ethanol (v/v), butanol, and ethyl acetate extracts than hexane, chloroform and water extracts (Lee et al., 2009). When using the ethanol, some precautions need to be taken, for example, the mixture of ethanol and

water has less volume than the sum of individual components at a given fraction, a mixture of ethanol and water is exothermic. Also over 50% ethanol (v/v) solutions are flammable.

Interestingly, the polyphenols extracts with ethyl acetate from natural materials were reported to have strong antioxidant capacity. Less polar solvents such as ethyl acetate provided more active extracts than ethanol or methanol, although ethanol and methanol extracts also presented high lipid peroxidation-inhibiting capacity (Moure et al., 2001).

### 2.3.4 The Effect of Extraction Temperature, Time and Solvent to Solid Ratio

The extraction is a function of how fast the compound is dissolved and the equilibrium is achieved by liquid solvents (Pinelo et al., 2006). The temperature has impact on solubility, diffusion coefficient (mass transfer rate) and the stability of phenolics compounds (Luthria, 2008). An increase in temperature and a decrease of viscosity significantly increase the diffusion rate. However, high temperature may degrade the phenolics. The driving force for the extraction is the concentration gradient within the particles, which is related to solvent: solid ratio. The rate of extraction increases with a larger concentration gradient (Cacace & Mazza, 2003).

A range of extraction temperatures and time were employed by researchers in the extraction of phenolic compounds from olive leaves. Generally less extraction time is required with the increasing temperature. When ethanol was used as a solvent, the extraction process took place either at room temperature (most frequently used) or elevated temperatures of 40° C for 24-48 hours under agitation (Bouaziz & Sayadi, 2005; Japon-Lujan et al., 2006; Jemai et al., 2008; Kiritsakis et al., 2010). A water bath was used to achieve required temperatures. With boiling water, 10 to 30 minutes extraction time was used by researchers (Pereira et al., 2007; Malik & Bradford, 2008). This kind of extraction is purely a static process and easy to operate. The solvent to solid ratio is normally expressed as the ratio of the volume of solvent (millilitre) to the weight of extraction sample (gram). The solvent to solid ratio employed by the researchers for olive leaf extraction varied hugely from 4 to 100, but a ratio between 10 and 50 was mostly reported in the literatures (Letutour, 1992; Savournin et al., 2001; Briante et al., 2002; Bouaziz & Sayadi, 2005; Silva et al., 2006; Pereira et al., 2007; Jemai et al., 2008; Malik &Bradford, 2008; Boudhrioua et al., 2009; Lee et al., 2009; Kiritsakis et al., 2010). Japon et al (2006) used 8ml solvent and 1gram of dried olive leaves in all their studies (Japon-Lujan et al., 2006a, 2006b).

### 2.3.5 The Effect of The Extraction pH

The pH of extraction medium determines the degree of solubility for soluble compounds and also influences the possible solubilisation of the hydrolysable fractions (Escribano-Bailon & Santos-Buelga., 2003). The influence of the pH of medium on the olive leaf extracts was examined by Japon et al. (2006a), and diluted aqueous solutions of hydrochloric acid and sodium hydroxide were used to achieve extraction at pH 2 and pH 12, respectively. By comparison of the extracts obtained at pH 2 and pH 12 with pH 8 showed that the amount of olive bioactive phenolics extracted at pH 12 decreased by 27% for apigenin-7-glucoside, 35% for oleuropein and luteolin-7-glucoside and 40% for verbacoside, probably due to the ester bond hydrolysis. However, the concentration of the target analytes at pH 2 was similar to those in the extract at pH 8 (Japon-Lujan et al., 2006a). Another study also indicated increased pH values were unfavourable for extraction of phenolic compounds from olive leaves (Mylonaki et al., 2008).

### 2.3.6 Purification of Crude Extracts

Liquid- liquid extraction (LLE) and solid phase extraction (SPE) are normally used as purification/ fractionation or pre concentration of phenolic compounds. One example of using liquid-liquid extraction to separate low-molecular weight polyphenols was given by Bouaziz & Sayadi (2005) as follows: the crude olive leaf extract was concentrated under vacuum to dryness, and then the residue was dissolved in methanol and extracted three times with ethyl acetate (Bouaziz & Sayadi, 2005). De Leonardis et al. (2008) developed a method to produce an olive leaf extract of high hydroxytyrosol content. The procedure involved an acid steam cooking of the integral olive leaf to directly hydrolyse the native complex phenols contained therein; successively, the phenols were recovered by a liquid-liquid extraction with ethyl acetate (De Leonardis et al., 2008).

Solid phase extraction with C18 cartidges has been used extensively for the fractionation of phenolic acid and flavonoids from crude extract. To avoid the oxidation of polyphenols, an antioxidant can be added (Escribano-Bailon & Santos-Buelga 2003). In order to obtain a good separation of peaks, Vinha et al. (2010) suggested that a purification of crude olive leaf extract using sep-pack C18 was recommended (Vinha et al., 2010).

Romani et al. (1999) developed a solid-liquid extraction (SLE) procedure with extrelut cartridge (diatomaceous earth) using different eluents to obtain polyphenolic compounds. By using this method, it was possible to detect and quantify several subclasses of

polyphenols, such as phenolic acids, oleuropein derivatives, flavons, flavonol glycosides, and anthocyanin derivatives using a small quantity of solvent (Romani et al., 1999).

Furthermore, silk fibroin was used as a novel adsorbent to recover the polyphenols from the olive leaf extracts. Silk fibroin was found to be a promising adsorbent for the purification of oleuropein and rutin from olive leaf extracts (Altiok et al., 2008).

### 2.3.7 Storage of Olive Leaf Extracts

The extracts need to be stored in a condition, which results in minimum loss of antioxidant capacity. Temperature and light are the major factors influencing antioxidant activity during storage. These methods protecting deteriorative processes include the use of inert atmospheres such as nitrogen and the absence of light (Tura & Robards, 2002). Although there were few studies regarding the stability of oleuropein, it was suggested that oleuropein was quite stable in methanol extracts for 30 days when stored at room temperature, and then started to degrade after that. In the aqueous extract, oleuropein was stable in the first 7 days and completely degraded after 17 days (Malik & Bradford, 2008). In previous studies, liquid olive extracts were normally stored at 0°C or 4°C in the dark, powdered extracts were stored at room temperature. The shelf life of commercial capsuled olive leaf extract is approximately two years at room temperature.

## 2.4 High-Performance Liquid Chromatography Analysis (HPLC) of Quantitative Determination of Phenolic Compounds in Olea europaea Leaf

### 2.4.1. HPLC Fundamentals

High performance liquid chromatography (HPLC) is one of the most powerful separation techniques, and it has been extensively used in the food industry for the analysis of components of both raw and processed products ( Nollet, 2000).The separation of the samples into individual components takes place when samples pass through a column carried by a flow of mobile phase. The separated compounds are eluted and detected by a detector, and presented as a set of chromatographic peaks in a chromatogram (Lunn, 2002).

A representative HPLC instrument consists of a mobile phase reservoir, a high pressure pump, an injection device, a separation column, a detector, and a data system. The separation column and detector are the heart of the HPLC system. The properties of the stationery phase and mobile phase such as polarity and size of particle determine the

mechanism of separation (Lunn, 2002). The sample is separated on the basis of solubility and polarity of the sample components. For normal phase HPLC the solvent (mobile phase) is non-polar and the column packing (stationery phase) is polar. Sample molecules are more or less attracted to the particles in the column as opposed to the solvent; the less polar molecules are eluted first. Revised-phase HPLC (RP HPLC) uses polar solvent and non-polar packing. In RP HPLC, the elution order is reversed (Nollet, 2000).

Isocratic elution and gradient elution are two common methods used in HPLC, in isocratic elution, mobile phase composition is held constant during the whole run, whereas, a gradient elution, mobile phase composition varies with test time. The most commonly used detector is a UV detector, which is based on the UV absorbance of eluting compounds. In diode array detectors (DAD) also known as photodiode array detectors, a series of given wavelengths can be simultaneously used (Lunn, 2002).

Before HPLC analysis, sample preparation normally extraction is often necessary to isolate the components of interest from a sample matrix. A pre-treatment may also be required, and which includes purification or fractionation step for removal of interfering compounds or an enrichment procedure in case of trace components. The sample pre-treatment can be achieved by liquid- liquid extraction (LLE) and solid phase extraction (SPE) on cartridges as mentioned previously (Nollet, 2000).

### 2.4.2 Methods For Separation of Phenolic Compounds in Olive Leaf Extract

For separation of phenolic compounds in olive leaf extract, the reversed-phase chromatography on silica-based C18 bonded-phase columns is the most reported. It was suggested by Bouaziz et al. (2004) that the C-18 column is more suitable for the resolution of the range of phenols and the C-8 column provided adequate separation of flavonoids (Bouaziz et al., 2004).

Aqueous acetic, formic, trifluoroacetic acid (TFA) or phosphoric acids with methanol or acetronitrile as an organic modifier are the solvents commonly used for the mobile phase. TFA (0.05%), 0.01% of phosphoric and formic acid, 0.2-5% of acetic acid were the acids adopted by most researchers. The pH and ionic strength of the mobile phase plays a crucial role in determining retention of phenolics on the column. The pH range most often used for RP-HPLC for phenolics is low, between 2 and 4. Small amounts of acetic acid or phosphoric acid are included in the solvent system and can improve the resolution and

reproducibility of analysis (Benavente-Garcia et al., 2000; Romani et al., 2001; Savournin et al., 2001; Vinha et al., 2002; Hayes et al., 2011).

The detectors used for HPLC analysis of olive leaf extract in previous studies were mainly photodiode array detector (PDA), absorbance at 280 nm, 340 nm and 350 nm were monitored for a variety of phenolic compounds (Japon-Lujan et al., 2006 ; Ranalli, et al., 2006; Goulas et al., 2010). UV-VIS detector and mass spectrometer were also used by many researchers (Laguerre et al., 2009). Gradient elution was adopted by most researchers for separation of phenolic compounds and external standard method were mostly used for quantification of those compounds in the olive leaf extracts (Benavente-Garcia et al., 2000; Romani et al., 2001; Savournin et al., 2001; Vinha et al., 2002; Hayes et al., 2011). The internal standard method was also used by some researchers to determine phenolic compounds in olive leaf extract. Savournin et al. (2002) used coumarin as an internal standard to determine oleuropein concentration. It was also recommended by Savournin et al. (2001) that the quantification of oleuropein using an internal standard is of particular importance because commercially available oleuropein standards are not of HPLC grade (Savournin et al., 2001).

## 2.5 The Role of Free Radical & Antioxidants in Biological & Food Systems

### 2.5.1 Free Radicals

A "*free radical*" is any species of substances that are capable of independent existence that contains one or more unpaired electrons (Halliwell et al., 1995). It is well known that free radicals generated in vivo can cause oxidative stress, which is an imbalance between reactive oxygen species (ROS)/reactive nitrogen species (RNS) and defence and repair systems, consequently causing damage to DNA, proteins and lipids in biological systems. This damage has been associated with human disease including atherosclerosis, rheumatoid arthritis and inflammatory bowel disease (Halliwell et al., 1995; Sanchez-Moreno, 2002). For food manufacturing, the oxidation of lipids caused by free radicals is a major concern, because it can lead to development of unpleasant "rancid" and "off-flavour" as well as potential toxic end products (Hallowell et al., 1995).

There are six major ROS and RNS that regularly interact and damage the major macromolecules in physiological and food-related systems: (1) the superoxide anion ($O_2^{\bullet -}$), (2) hydrogen peroxide ($H_2O_2$), (3) the peroxyl radical ($ROO^{\bullet}$), (4) the hydroxyl radical

($OH^{\bullet}$), (5) singlet oxygen ($^1O_2$), and (6) peroxynitrite ($ONOO^-$). Of all these radicals, the peroxyl radical ($ROO^{\bullet}$) has been most intensively studied as it is a key component of auto-oxidation (MacDonald-Wicks et al., 2006).

For example, exposure of organism to ionizing radiation has been known to favour development of cancer, it may partially be due to formation of the hydroxyl radical ($OH^{\bullet}$) in the body causing direct DNA damage. When the superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$) react with low density lipoprotein (LDL) in a cell membrane, the resulting per oxidation of LDL contributes to atherosclerosis. In food system, the peroxyl radical ($ROO^{\bullet}$) is a major free radical resulting in the lipid peroxidation and consequent rancidity in the oil and the foods (Gutierrez-Rosales et al., 1998).

### 2.5.2 Dietary Antioxidants and Their Source

Antioxidant can significantly delay or prevent oxidation of targeted substrate by scavenging free radicals. A dietary antioxidant is "a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in human beings" (Huang et al., 2005). A dietary antioxidant is a non-enzymatic antioxidant, which often broadly includes radical chain inhibitors (e.g. vitamins C and E), metal chelators (e.g. Ethylenediaminetetraacetic acid-EDTA), oxidative enzyme inhibitors (e.g. aspirin), and antioxidant enzyme cofactors (e.g. Se). A dietary antioxidant can scavenge reactive oxygen/nitrogen species (ROS/RNS) to stop radical chain reactions, or it can inhibit the reactive oxidants from being formed in the first place (Huang et al., 2005). Diet-derived antioxidants are primarily plant phenolic compounds that may occur in all parts of plants and function as radical chain inhibitors.

Phenolic compounds are plant secondary metabolites that possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer. The latter groups of compounds are often referred to as polyphenols. The phenolic acids, flavonoids and tannins are three major groups of phenolic compounds. The antioxidant activity of phenolic compounds depends on the structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings (Balasundram et al., 2006).

Fruits and vegetables and related products such as juice and wine are major sources of natural antioxidants in the diet. Antioxidant compounds present in fruits and vegetables are

mainly flavonoids, phenolic acids, tocopherols, carotenoids, ascorbic acid and sulphur-containing compounds. Other sources of natural antioxidant are herbs, spices and teas where a large diversity of phenolic compounds have been found in those foods (Yanishlieva-Maslarova, 2001). Furthermore, polyphenols, phenolic acid and tocopherols are the compounds that contribute to antioxidant capacity of legumes, nuts and beans.The antioxidants common to oilseeds include tocopherols and tocotrienols (Hall, 2001).

Synthetic phenolic antioxidants that have been most frequently used in food as additives are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ); due to safety concerns of those antioxidants, both consumers and manufacturers are clearly showing preference towards natural antioxidants in recent years (Moure et al., 2001). To replace and reduce synthetic antioxidants in food applications, the attempt to recover bioactive compounds from agricultural by-products has gained increasing attentions (Moure et al., 2001; Balasundram et al., 2006). Balasundram et al. (2006) indicated that agricultural industry by-products could be a good source of naturally occurring antioxidants. Some examples of those by-products are grape seeds, residues from olive oil industry and apple peels (Balasundram et al., 2006).

### 2.5.3 Auto-Oxidation and Its Inhibition

Huang et al. (2005) summarised that a typical auto-oxidation reaction initiated by an azo compound and its inhibition by an antioxidant include the following elementary steps shown as Figure 2.1 (Huang et al., 2005).

The classical route of autoxidation includes initiation (production of free radicals), propagation and termination (production of nonradical products) reactions. Phenolic antioxidants (AH) function as a sacrificial agent by rapid donation of a hydrogen atom to radicals and preventing the primary oxidation. This reaction competes with two chain propagation reactions (step 6 & step 7), and a good and more effective antioxidant should react with the peroxyl radical (step 6 in Figure 2.1) much faster than reaction with the substrate (LH) (step 3 in Figure 2.1) (Huang et al., 2005).

Initiation

1) $R_2N_2 \rightarrow 2R + N_2$
2) $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$
3) $ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$

Propagation

4) $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$
5) $LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$

Inhibition

6) $ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$
7) $LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$

Terminaiton

8) $A^{\bullet} + (n-1)\ LOO^{\bullet} \rightarrow$ nonradical products
9) $LOO^{\bullet} + LOO^{\bullet} \rightarrow$ non radical products

Figure 2.1: The Process of Auto-Oxidation and Its Inhibition (Sourced from Huang et al., 2005)

- $R_2N_2$ : azo compound
- $R^{\bullet}$: free radicals $ROO^{\bullet}$: the peroxyl radical
- LH: substrate e.g. low-density lipoproteins (LDL)
- AH: antioxidant e.g. α-tocopherol or phenolic compounds

### 2.5.4 Common Methods for the Determination of Antioxidant Capacity

The most common methods used for the determination of antioxidant capacity in foods and biological systems have been discussed by many researchers. However, none of the methods have been suggested to be the most convenient and appropriate method such method could be regarded as a standard method for measurement of antioxidant capacity. Hence, there are a number of different methods. Even with terms used to express how capable an antioxidant can protect a biological system or food system against the potential harmful effects involving ROS and RNS, different researchers have different views (Karadag et al.,2009). Huang and co-workers (2005) suggested it would be more appropriate to use oxidant-specific terms such as “peroxyl radical scavenging capacity” to describe the result from specific assays rather than only use ‘total antioxidant capacity” (Huang et al., 2005). The term “antioxidant activity” would be meaningless without the context of specific reaction conditions. Macdonald et al. (2006) indicated that different meanings of antioxidant activity and antioxidant capacity should be recognised although

those two terms are often used interchangeably. Activity refers to the rate constant of a reaction between a specific antioxidant and a specific oxidant. Capacity is a measure of the amount (in moles) of a given free radical scavenged by an antioxidant (MacDonald-Wicks et al., 2006).

Ideally, the antioxidant capacity should be tested using both in-vitro and in-vivo techniques, but due to the high cost of in vivo test, many products undergo only in vitro testing (MacDonald-Wicks et al., 2006). Sanchez (2002) also suggested when determining the effectiveness of the dietary antioxidant in the function of disease prevention, the evaluation of the real contribution of the antioxidant to antioxidant status in biological systems must be evaluated (Sanchez-Moreno, 2002).

In this part of the literature review, six common in vitro assays and their strengths and limitations are discussed, and antioxidant capacity is used as the term to describe the result obtained from the different assays.

### 2.5.5 Classification of Antioxidant Capacity Assays

Sanchez (2002) classified the antioxidant tests into two categories: those assays used to evaluate lipid peroxidation, in which a lipid or lipoprotein substrate under standard conditions is used and the degree of oxidation inhibition is measured, and those assays used to measure free radical scavenging ability. The essential features of any tests are a suitable substrate, an oxidation initiator and an appropriate measure of the end-point that could be chemical, instrumental or sensory methods, and most of the assays belong to the second group (Sanchez-Moreno, 2002). An alternative classification of antioxidant capacity assays is based on hydrogen atom transfer (HAT) reactions and single electron transfer (SET) (Huang et al., 2005; Prior, Wu & Schaich, 2005). Sanchez's (2002) classification is mainly based on methodology, whereas Huang et al. (2005) and Prior et al. (2005) looked into the chemistry behind these assays, and it appeared the latter classification was more accepted by many food chemists. According to Huang et al. (2005) classification, the major HAT based assays and ET-based assays are shown in Table 2.4.

In general, ET based assays involve the redox reaction with the oxidizing agent such as Fe as a probe to monitor the reaction and an indicator of reaction endpoint. In contrast, most HAT based assays monitor competitive reactions kinetics, and the quantitation is derived

from the kinetic curve. HAT based assays are generally composed of a synthetic free radical generator, a molecular probe, and an antioxidant. Both of these methods are intended to measure the radical (or oxidant) scavenging capacity (Huang et al., 2005).

Table 2. 4: In Vitro Antioxidant Capacity Assays (Sourced From Huang Et Al., 2005)

| 1) HAT Based Assays | 2) ET Based Assays |
|---|---|
| **ORAC**(oxygen radical absorbance capacity) | **TEAC** ( Trolox equivalent antioxidant capacity) |
| **TRAP**(total radical trapping antioxidant parameter) | **FRAP** ( Ferric reducing antioxidant capacity) |
| Crocin bleaching assay | **DPPH** (diphenyl-1-picrylhydrazyl) |
| IOU(Inhibition Oxygen uptake) | Copper II reduction capacity |
| Inhibition of linoleic acid oxidation | **Total phenols assay** by Folin-Ciocateu reagent |
| Inhibition of LDL oxidation | |

### 2.5.6 ET Based Assays

ET based assays detect the ability of a potential antioxidant to donate or transfer an electron to reduce any compounds including metals, carbonyls and radicals. ET based reactions are pH independent, usually slow and can require a long time to reach completion (Prior et al., 2005).

"The reaction mechanism can be described by this reaction"
Probe (Oxidant) + e (from antioxidant)⟹reduced probe +oxidised antioxidant"
(MacDonald-Wicks et al., 2006)

### TEAC (Trolox Equivalent Antioxidant Capacity)

The TEAC assay was developed by Miller et al. (1993) and Re et al. (1999) and has been used widely in testing antioxidant capacity in food samples (MacDonald-Wicks et al., 2006). This assay measures the relative abilities of antioxidants to scavenge radicals in comparison with the antioxidant potency of a standard antioxidant compound 6-hydroxy-2,-5,-7,-8-tetramethylchroman-2-carboxylic acid (Trolox) (Zaporozhets et al., 2004).

Trolox is a water soluble derivatives of α-tocopherol and commonly used as a reference free radical scavenger. The original ABTS assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS (2, 2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid) and antioxidants to produce the radical cation. The ferrylmyoglobin radical is generated first and then oxidizes the ABTS, forming the ABTS radical cation (Miller et al., 1993). The $ABTS^{\cdot+}$ formed in this way has a strong absorption in the range of 600–750 nm and can be easily determined spectrophoto-metrically. The absorbance of the reaction mixture is read when the colour of the incubation mixture is stable or at a fixed time point (Roginsky & Lissi, 2005). This has been criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferrylmyoglobin radical (Miller et al., 1993).

An improved TEAC assay proposed by Re et al. (1999) can be applicable to both lipophilic and hydrophilic antioxidant in aqueous and lipophilic systems. Compared with the original TEAC assay, this decolourization assay is improved in the way that free radical cation $ABTS^{\cdot+}$ are pre-formed in a stable form through reaction between ABTS and potassium persulfate prior to addition of the antioxidant, rather than the generation of the radical continually in the presence of the antioxidant. The $ABTS^{\cdot+}$ is blue colour and can be reduced to its colourless neutral form after its reaction with an antioxidant. Therefore, the antioxidant capacity is determined by decolourization of $ABTS^{\cdot+}$, which is measured though the percentage of reduction of absorbance at 734nm at defined time points for antioxidants at different concentration. The antioxidant activity can be described in two ways: TEAC value (Trolox equivalent antioxidant activity) and AUC value (relative antioxidant activity) (Re et al., 1999). It may be carried out using RANDOX kit (Randox Laboratories Ltd, Ardmore, UK) (Zaporozhets et al., 2004).

**FRAP (Ferric Reducing Antioxidant Capacity)**

The FRAP assay is based on the ability of phenolics to reduce yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants .The resulting blue colour measured spectrophotometrically at 593 nm is taken as linearly related to the total reducing capacity of electron-donating antioxidants. The FRAP assay needs acidic (pH 3.6) conditions to

maintain the iron solubility. One FRAP unit is defined as the reduction of 1 mol of Fe (III) to Fe (II) (Karadag et al., 2009).

**DPPH (2.2-diphenyl-1-picrylhydrazyl)**

The DPPH assay is the oldest method for determining antioxidant activity and 2.2-Diphenyl-l-picrylhydrazyl (DPPH$^{\cdot}$) is one of a few stable and commercially available organic nitrogen radicals (Huang et al., 2002; Roginsky & Lisiis, 2005); it has absorption at 515nm and disappears upon reduction by an antioxidant. By measuring the decrease in absorbance at 515nm for different concentrations of antioxidants (expressed as the number of moles of antioxidants or mole DPPH free radicals) over a time period of normally 30 minutes, the reaction kinetics can be obtained by plotting the percentage of remaining DPPH free radical at steady state against time; the antioxidant capacity is expressed as EC 50 which is defined as the amount of antioxidant necessary to decrease the initial DPPH free radical concentration by 50%, the smaller EC 50 the value is, the more efficient the antioxidant is (Brandwilliams et al., 1995).

**Total phenols assay by Folin-Ciocateu Reagent (FCR assay)**

The FCR assay is based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic and phosphotungstic acids. After oxidation a green–blue complex is formed and measured spectrophotmetrically at 760 nm. The total phenolic content is expressed as gallic acid equivalents if gallic acid is used as the standard (Schlesier et al., 2002). Ainsworth & Gillespie (2007) proposed a protocol of estimation of total phenolic content using Folin-Ciocalteu reagent. The FRC assay provides a general diagnostic tool of the antioxidant capacity of leaf tissue extracts (Ainsworth & Gillespie, 2007).

### 2.5.7 Advantages and Disadvantages of ET Based Assays

All ET based assays discussed above are indirect methods; all assays actually characterize the capacity of the tested sample to react with radical cations rather than to inhibit the oxidative process. TEAC is the most popular assay amongst these assays. The advantage of the TEAC test is its relative simplicity that allows its application for routine determinations in any laboratory. However, the TEAC assay may be time consuming as the reaction

between phenolics and ABTS is rather slow (Roginsky & Lissis, 2005).

In contrast, the FRAP is one of the most rapid tests and very useful for routine analysis, by using micro plates, a lot of samples can be analysed within a short time. The limitation of the FRAP is the non-physiologically low pH value (3.5) required (Schlesier et al., 2002). One of major disadvantages of the FRAP assay is that metal chelators in food extracts may bind Fe and form complexes that are also capable of reacting with the antioxidant; this may cause problems and overestimate the antioxidant capacity. Polyphenols including caffeic acid, tannic acid, ferulic acid and quercetin cannot be measured accurately if a four minutes reaction time is followed in the FRAP assay due to incomplete reactions (Huang et al., 2002). Karadag et al. (2009) claimed that too many different end points of FRAP assay have been used which may cause great problem. The use of the lag time alone often underestimates antioxidant capacity considerably (Karadag et al., 2009). Furthermore, FRAP is caused by the bromines ability to take part not only in redox or radical reactions but also in the addition and substitution reactions that may also cause problem and inaccurate results (Zaporozhets et al., 2004).

Sanchez (2002) recommended that DPPH method is an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable extracts. The results are highly reproducible and comparable to other free radical scavenging methods such as TEAC assay. However, this method is not useful for measuring the antioxidant activity of plasma, because protein is precipitated in the alcoholic reaction medium (Sanchez-Moreno, 2002). This assay is also not a competitive reaction because DPPH is a stable nitrogen radial that bears no simility to the highly reactive and transient peroxyl radicals. Many antioxidants that react with quickly with peroxyl radical may react slowly or may even be inert to DPPH due to steric inaccessibility (Prior et al., 2005).

The FCR assay actually measures reducing capacity, thus there is always the controversy over what is being detected in total antioxidant capacity assays- only phenols, or phenols plus reducing agents plus possibly metal chelators. However, the FCR assay is convenient, simple, and reproducible, is becoming a routine assay in studying phenolic antioxidants (Huang et al., 2002; Prior et al., 2005).

### 2.5.8 HAT Based Assays

HAT based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation; HAT based reactions are solvent and pH independent and are usually quite rapid, typically completed in seconds to minutes ( Prior et al.,2005). In general, these assays provide a steady flux of peroxyl radicals from a radical initiator – usually 2, 2'-azobis (2-amidinopropane) hydrochloride (AAPH) in air saturated solutions in the presence of a molecular probe that fluoresces when oxidized. Added antioxidants compete with the probes for oxidation. The antioxidant capacity is measured by reduction of intensity of fluorescent (MacDonald-Wicks et al., 2006). Two methods, the ORAC and TRAP assays are discussed in this section, and both assays are direct competition methods (Roginsky &Lissi, 2005).

**ORAC (Oxygen Radical Absorbance Capacity)**

The ORAC measures antioxidant inhibition of peroxyl-radical induced oxidants and reflects classical radical chain breaking antioxidant activity by hydrogen atom transfer (Ou et al., 2001).

$$R{-}N{=}N{-}R \xrightarrow{O_2} N_2 + 2ROO^{\bullet}$$

$$ROO^{\bullet} + \text{probe (fluorescent)} \rightarrow \text{ROOH} + \text{oxidized probe (loss of fluorescence)}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

$$ROO^{\bullet} + A^{\bullet} \xrightarrow{fast} ROOA$$

Figure 2. 2: The chemical mechanism behind ORAC assay (sourced from Prior et al., 2005)

As shown in Figure 2.2, the peroxyl radical reacts with a florescent probe to form a non fluorescent product (oxidized probe), which can be quantified by florescence. Antioxidant capacity is determined by a decreased rate of peroxyl radical and amount of oxidized probe formed over time. The peroxyl radicals are generated by AAPH˙ and fluorescein at constant temperature 37°C. ORAC values are expressed as micromoles of Trolox equivalent (TE) per litre or per gram of sample (Prior et al., 2005).

A fluorescent probe typically possesses conjugated double bond structure that give rise to

their fluorescent properties as well as susceptibility to react with free radical that results in nonfluorescent product. The rate of decay of fluorescent intensity, therefore, reflects the rate of peroxidation (Arti-Arora, 1997). Fluorescein (FL) is the currently preferred fluorescent probe because it is more stable and less reactive than B-phycoerythrin (B-PE) which was employed in the first version of ORAC (Ou et al., 2001). However, FL is pH sensitive and this must be carefully monitored (MacDonald-Wicks et al., 2006). The ORAC assay has been used not only in evaluation of antioxidant capacity of fruits and vegetables, dietary supplements, wines, juices, and nutraceuticals, but also in plasma or serum samples (Karadag et al., 2009).

**TRAP (Total Radical Trapping Antioxidant Parameter)**

The TRAP assay was initially introduced to measure the total antioxidant status of human plasma. This assay is based on the ability of plasma to trap a flow of peroxyl radicals at a constant rate. The peroxyl radicals are generated at a controlled rate by the thermal decomposition of $AAPH^{\cdot}$ or $ABAP^{\cdot}$ in aqueous buffer at 37°C (Sanchez et al., 2002). The TRAP assay uses R-phycoerythrin (R-PE) as a fluorescent probe, the intensity of fluorescence of R-PE decreases with time under the flux of the peroxyl radicals. In the presence of a tested sample containing antioxidants, the decay of R-PE fluorescence is retarded and the reaction progress is monitored fluorometrically ($\lambda_{excitation}$ = 495nm and $\lambda_{emission}$ = 575nm) (Sanchez et al., 2002; Roginsky et al., 2005; Karadag et al., 2009). This is compared to that of Trolox and then quantitatively related to the antioxidant capacity of plasma. The TRAP assay has been also used in vitro to evaluate the antioxidant activity of beverages and food such as green tea and red wine (Sanchez et al., 2002).

In summary, ORAC and TRAP assays can be applied for both hydrophilic and lipophilic chain breaking antioxidant capacity against peroxyl radicals. The major difference amongst these two assays is the quantitation approaches; ORAC assay determines the area under the kinetic curve (AUC), whereas the TRAP relies on lag time. The advantage of AUC approach is that it applies equally well for both antioxidants that exhibit distinct lag phase and no lag phase. Also different molecular probes are used; the TRAP assay uses R-PE and ORAC uses FL, both methods use AAPH as a radical initiator (Huang et al., 2002)

### 2.5.9 Advantages and Disadvantages of HAT Based Assays

In respect of advantages of the two assays, firstly the ORAC assay provides a controllable source of peroxyl radicals that simulates reactions of antioxidants with lipids in both food and physiological system, and it can be adopted to detect both hydrophobic and hydrophilic antioxidants by altering the radical source and solvent (Prior et al., 2005). Secondly the ORAC assay measures the protective effect of an antioxidant by the net integrated area under the fluorescence decay curve of the sample compared to that of a blank (AUC sample-AUC blank), which covers lag phase, initial rate and total imbibitions, and thus it avoids underestimation of antioxidant capacity. It is particularly useful for samples that contain multiple ingredients and have complex reaction kinetics (Karadag et al., 2009).

Sanchez et al. (2002) pointed out the main disadvantage of the ORAC and TRAP method is that it is assumed that the oxidative deterioration and the antioxidant mechanism can mimic critical biological substrates such as plasma and protein sample (Sanchez et al., 2002). The assumption of ORAC assay principle and the troublesome process have been a major criticism for this assay (Prior et al., 2005). Huang et al. (2002) proposed an enhanced high-throughput assay by employing a robotic eight-channel liquid handling system along with a micro plate fluorescence reader; the improved method enhanced the efficiency of the assay with at least a 10-fold increase in sample throughput over the current procedure (Huang et al., 2002). However the ORAC reaction is temperature sensitive, and thus close temperature control throughout the plate is essential to achieve accurate result (Prior et al., 2005).

### 2.5.10 Comparison of Methods

The following two tables compare assays discussed above with respect to their limitations and strengths. It can be seen from Table 2.5 and Table 2.6, the ORAC and TRAP are the most biological relevant assays, and ORAC is relatively simpler than TRAP. ET based assays are simple to measure compared to HAT based assay, and FRAP is the most convenient assay but the least biological relevant. ORAC and TEAC are adaptable to measure both lipophilic and hydrophilic antioxidants, whereas FRAP is best used for lipophilic antioxidant, and DPPH is more suitable for hydrophilic antioxidants. The quantitation methods of FRAP, TEAC and DPPH are similar, which use a fixed time or inhibition degree as the endpoint, and thus the time selection is critical to the assays (Prior

et al., 2005).

Table 2. 5: Comparison of Methods for Assessing Antioxidant Capacity

| Antioxidant assay | Simplicity | Instrumentation required | Biological relevant | Mechanism | Endpoint | Quantitation | Lipophilic and hydrophilic AOC |
|---|---|---|---|---|---|---|---|
| ORAC | ☺☺ | ☺ | ☺☺☺ | HAT | Fixed time | AUC | ☺☺☺ |
| TRAP | ☹☹☹ | ☹☹specialized | ☺☺☺ | HAT | Lag phase | IC50 lag time | ☹☹ |
| FRAP | ☺☺☺ | ☺☺☺ | ☹☺ | SET | Time, varies | ΔOD fixed time | ☹☹ |
| TEAC | ☺ | ☺ | ☹ | SET | Time | ΔOD fixed time | ☺☺☺ |
| DPPH | ☺ | ☺ | ☹ | SET | IC50 | ΔOD fixed time | ☹ |

☺,☺☺,☺☺☺ =desirable to highly desired characteristic

☹,☹☹,☹☹☹=less desirable to highly undesirable based on this characteristic

Reproduced from (Prior et al., 2005)

Table 2.6: Methods for Assessing Antioxidant Capacity Compared By Key Criteria

| | Principle | |
|---|---|---|
| | Hydrogen atom transfer | Electron transfer |
| Examples | ORAC<br>TRAP | Total phenols<br>TEAC<br>DPPH |
| Biologically relevant? | Yes | No |
| Simple to measure? | No (except ORAC) | Yes |
| Instrumentation readily available? | No | Yes |
| Reproducible? | Undetermined | Undetermined |
| Suitable for hydrophilic and lipophilic antioxidants? | No (except ORAC) | No (except TEAC) |

Reproduced from (MacDonald-Wicks et al., 2006)

### 2.5.11 Summary of Antioxidant Capacity Assays

Due to the differences between the testing systems investigated, it is strongly suggested to use at least two methods depending on the antioxidant potential expected and perhaps on the origin of substances (Schlesier et al., 2002). Electron transfer and hydrogen atom

transfer are two mechanisms behind different antioxidant capacity assays, and hydrogen atom transfer reaction apparently is a key step in the radical chain reaction. Therefore, HAT based method is more relevant to the radical chain breaking antioxidant capacity. On the basis of this analysis, Huang et al.(2002) suggested that the total phenol assay by FCR could be used to quantify an antioxidant's reducing capacity and the ORAC assay to quantify peroxyl radical scavenging capacity (Huang et al., 2002). Prior et al. (2005) also proposed that ORAC, FCR and possibly TEAC be considered for standardization. The Folin-Ciocalteu method is an ET based assay and gives reducing capacity, which has normally been expressed as phenolic contents. ORAC represents a hydrogen atom transfer HAT reaction mechanism, which is most relevant to human biology.

It is also important to know that almost all in vitro assays measure the radical scavenging capacity or reducing ability, respectively, not the preventative antioxidant capacity of a sample (MacDonald-Wicks et al., 2006). No single assay can be considered a "total antioxidant capacity assay" even though it can be performed both in an aqueous solution and in a lipophilic environment. To fully describe a full profile of antioxidant capacity against various ROS/RNS, different methods specific for each ROS/RNS may be needed (MacDonald-Wicks, et al., 2006)

## 2.6 Phenolic Compounds in the Olive Leaf and Their Antioxidant Capacity

### 2.6.1 Major Phenolic Compounds in Olive Leaf

There are five groups of phenolic compounds present in olive leaves, namely oleuropeosides (oleuropein and verbascoside), flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin, and diosmetin), flavonols (rutin),flavan-3-ols (catechin), and substituted phenols (tyrosol,hydroxyrosol,vanillin, vanillin, vanillic acid, and caffeic acid) (Benavente-Garcia et al., 2000). The antioxidant activity of flavonoids present in olive leaves are influenced by the presence of functional groups in their structure, mainly the B-ring catechol, the 3-hydroxyl group and the 2, 3-double bond conjugated with the 4-oxo function. An example of apigenin-7-glucoside is shown as in Figure 2. 3.

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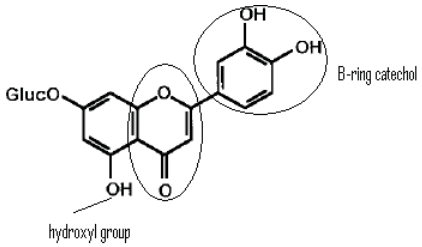


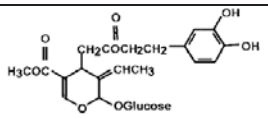
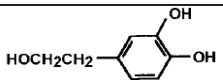
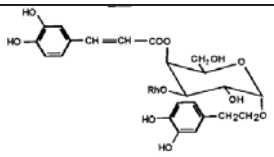
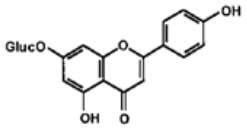
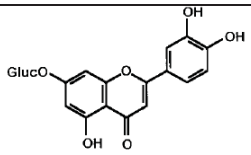

Figure 2.3: The Chemical Structure of apigenin-7-glucoside

For other phenolic compounds, the antioxidant activity is mainly influenced by the number and position of free hydroxyl groups in their structure. The five most abundant phenolic compounds present in olive leaves and their structure and is given in Table 2.7, of all the phenolic compounds, oleuropein is the most abundant one in olive leaves (Benavente-Garcia et al., 2000; Japon-Lujan & de Castro, 2006).

Oleuropein is present throughout the olive tree including all parts of fruit, but most of it is in the leaves. Oleuropein in olive oil ranges between 0.005% and 0.12%, in alperujo up to 0.87% by weight, and in leaves between 1 and 14%. Alperujo is the semisolid residue resulting from olive oil extraction; it is a major waste stream from the olive oil industry and is also a cheap source of natural antioxidants (Japon-Lujan et al., 2006a). The levels of oleuropein varied in the published literatures due to different varieties and extraction methods used. Soler et al. (2000) reported in the review that the 19% ( w/w) oleuropein and 1.8% ( w/w) flavonoid, of which 0.8% is luteolin 7-glucoside present in the olive leave, but there was 24.45% (w/w) oleuropein and 1.38% (w/w) of luteolin 7-glucoside found in olive leaf by Benavente-Garcia and co-workers (Benavente-Garcia et al., 2000; Soler-Rivas et al., 2000).

Oleuropein belongs to a special group of phenolic compounds called “secoirdoids” which are produced from the secondary metabolism of terpenes as precursors of indole alkaloids, and are usually derived from the oleoside type of glucosides, which is a combination of elenoic acid and a glucosidic residue. This type of compounds is exclusive to the *Olea* family (Silva et al., 2006).

Table 2.7: Chemicals Structures of the Most Abundant Phenolics in Olive Leaf Extract; Reproduced From (MacDonald-Wicks, et al., 2006)

| **Phenolic Compounds** | **Chemical Formula** | **Chemical Class** |
|---|---|---|
| Oleuropein |  | Secoirdoids |
| Hydroxytyrosol |  | Phenolic alcohol |
| Verbascoside |  | Flavonoids |
| Apigenin-7-glucoside |  | Flavonoids |
| Luteolin-7-glucoside |  | Flavonoids |

Hydroxytyrosol is a phenolic alcohol and one of the derivatives of oleuropein, and it has demonstrated good antioxidant properties. Unlike oleuropein which is present in high amounts in unprocessed olive fruits and leaves, hydroxytrosol is more abundant in the processed fruits and olive oil. The decrease of oleuropein and the increase in the concentration of hydroxytrosol occur due to chemical and enzymatic reactions that take place during the maturation of the fruit or as a result of olive processing (El & Karakaya, 2009). Hydroxytrosol can also be isolated by adding acid to hydrolyse the native complex phenols such as oleuropein in the olive leaves and recovered through liquid-liquid extraction (De et al., 2008). The principle behind this action is to break the ester bond of oleuropein and release the hydroxytyrosol. Briante et al. (2002) also reported a very simple and fast method to collect eluates with high amounts of hydroxytrosol, bio transforming olive leaf extract by a thermophilic ß- glucosidase immobilized on chitosan in a bioreactor (Briante et al., 2002). Verbascoside is a glycoside of caffeic acid and hydroxytrosol, which is also almost ubiquitous in the Olea family.

The phenolic compositions of olive fruits and leaves were found to be very complex and depend on a number of factors such as maturation stage and cultivar (Soler-Rivas et al., 2000). The oleuropein is very abundant in the early stage in young fruits, and decline rapily during maturation. Small fruit cultivars are characterized by high oleuropein and low verbascoside contents, while large fruit cultivars are characterized by low oleuropein and high verbasoside content (Soler-Rivas et al., 2000). Another study on polyphenol oxidase and its relationship with oleuropein concentrations in fruits and leaves of 'Picual' olive trees during fruit ripening discovered that polyphenol oxidase activity increased significantly during fruits maturation and oleuropein concentration decreased in the fruits and increased in the leaves. Polyphenol oxidase is an enzyme presents in the plant those catalyses the oxidation reaction of phenols resulting in fruit browning (Ortega-Garcia et al., 2008).

Genetic factors, age or colour of leaves were reported as important factors affecting the content of oleuropein in olive leaves. Seven common Italian cultivars have been investigated by Ranalli et al. (2006) and they found that genetic factor significantly affected oleuropein content in olive leaves. For instance, 'Nebbio' has the much higher content of oleuropein (8.55 g/kg fresh leaves) in the leaves than 'Leccino' (2.44 g/kg fresh leaves) (Ranalli et al., 2006). Another study investigated olive leaves of 14 different cultivars and found that 'Bid el Hama', 'Chemlali', 'Meski', 'Cailletier', 'Tanche', a 'Verdale-Picholine hybrid' and 'Lucques', in particular, had high oleuropein concentration and could be useful for sources for industrial extractions (Savournin et al., 2001).

The colour of leaves is another important factor, intense green colour including those at the developing stage have the highest content of oleuropein and antioxidant potency, while the green-yellowish and noticeably yellow leaves had noticeably lower content of oleuropein. For 1000 g fresh 'Leccino' leaves, there was only 1.1g oleuropein detected in yellow leaves compared with 2.44 g in green leaves. This suggested oleuropein in olive leaves is gradually degraded with the aging and is due to endogenous ß–glucosidase enzyme activity. The oleuropein in olive leaves is generally not influenced by the collection period; there is no substantial difference between leaves collected in spring and autumn (Ranalli et al., 2006).

### 2.6.2 The Antioxidant Capacity of Phenolic Compounds in Olive Leaf Extracts

Many different assays have been adapted by researchers to determine the antioxidant capacity of olive leaf extract such as DPPH, and TEAC, which makes it difficult to compare the results between different methods. Benavente et al. (2000) provided antioxidant capacity of each individual phenolic compound in olive leaf extract using TEAC method, the order of relative radical scavenging abilities was found to be: "Rutin>luteolin >olive leaf extract ~ hydroxytyrosol>caffeic acid>verbascoside >oleuropein>luteolin-7-glucoside~vannillic acid> apignin-7-glucoside>tyrosol"

The olive phenolics exhibited a synergic behaviour when mixed, as occurs in olive leaf extracts (Benavente-Garcia et al., 2000). However, a recent study shows the DPPH radical scavenging activity of olive leaf phenolics are in a slightly different order as follows (Lee & Lee, 2010):
"Caffeic acid>olive leaf extract>rutin>oleuropein>vanillin"

Despite these different results, all research are in agreement with the fact that olive leaf phenolics are much more effective than BHT and vitamin E with regards to their antioxidant capacity (Letutour & Guedon, 1992; Romani et al., 1999). Another study reported that the contribution of flavonoids to the overall DPPH radical scavenging of olive leaf extract is 13-27% and luteolin-7-glucoside was one of main scavenging phenolics (Goulas et al., 2010). Generally, the antioxidant capacity of leaf extracts and the total phenol content showed a positive correlation. However, a phenolic profile should also be considered as important in relation to total antioxidant capacity of olive leaf extract.

## 2.7 Industrial Process of Olive Leaf Extracts

Two processes have been patented for the extraction of oleuropein from olive leaves (Nachman, 1998; Postma & Erickson, 2005). These extracts are mainly characterized as health supplements. Both methods involves covering the leaves with the aqueous alcohol solution for a period of time, the extractions are conducted at different temperatures. One of patents (Nachman, 1998) uses the fresh leaves for extraction; the fresh leaves are used with at least 25% ethanol by weight for over four hours at a temperature range of 20°C to 88°C. The process is repeated twice and the extracts are concentrated through distillation

under vacuum, and then dried by spray drying or oven drying. The oleuropein content in powder extracts obtained by this method was claimed to be 30% to 40% by weight (Nachman, 1998). Another patent described a procedure for obtaining olive leaf extract from dried leaves. The 106 kg fresh leaves were dried in a forced air dryer at a temperature of less than 35°C, and the dried leaves was crushed by a cryogenic process. The extraction was carried out with 600 litres of 95% methanol of at a temperature of less than 20°C for 18 hours. Then filtered liquid extracts were concentrated in a vacuum at a temperature of less than 40°C, the extracts were then purified by sterile filtration. The aqueous solutions were dried into powders by freezing drying; the powdered extract contains 21.9% oleuropein and 5% flavonoids by weight (Almagro et al., 2004). Besides the extraction process, Postma & Erickson (2005) also invented a method to preserve fresh olive leaves before making up extract by soaking leaves in ascorbic acid water solution (Postma & Erickson , 2005)

## 2.8 Conclusions

The olive tree (*Olea europaea*) is one of the most important fruit trees in the world; Olive fruits are consumed as table olives, or used for extraction of olive oil. The published research indicates that consumption of olives and related products has been associated with prevention of diseases and can inhibit against pathogenic microorganisms. The nutritional and health benefits of olives and relevant products are attributed to constitute of the olives fruits and leaves, in particular, various phenolic compounds. The phenolic compound well reported to have scavenging abilities against free radicals. Free radicals can result in oxidative stress, consequently causing damage to DNA, proteins and lipids in biological systems.

One of most important phenolic compounds in the olive leave is oleuropein, which is present throughout the olive tree including all parts of fruit, but most of it is in the leaves. The phenolic compositions of olive fruits and leaves were found to be very complex and depended on a number of factors such as maturation stage and cultivar, and they undergo changes during the development of olive fruits. The cultivar and colour of olive leaves are important factors that affect the levels of oleuropein in the leaves.

Olive leaves are by-products of tree pruning and olive oil production; the utilization of

olive leaves in making olive leaf extract has drawn increasing attention. Solvent extraction was the main method adopted by most researchers to extract phenolics from olive leaves. The extraction solvent, temperature and time are important parameters to ensure high recover of phenolic compounds during the extraction process. The anti-oxidative capacity of phenolic compounds can be evaluated through different assays; Due to the differences between the testing systems investigated, it was strongly suggested to use at least two methods depending on the antioxidant potential expected and perhaps on the origin of substances.

Solvent extraction could be considered as a method for recovering phenolic compounds from olive leaves. The method needs to be relatively simple and quick. A non-toxic solvent is required for extraction and a cheaper solvent would be advantagous from a cost consideration. The critical extraction parameters need to be investigated in order to achieve good recovery of phenolic compounds. The other factors which affect extraction yields such as cultivars of olive leaves and sample preparation methods should also be concerned.

Reversed-phase high performance liquid chromatography (HPLC) on a silica-based C18 bonded-phase column is the most reported in previous studies for identification and quantitation of phenolic compounds in olive leaf extracts.  A purification of crude leaf extracts may be required prior to HPLC in order to obtain good peak separation.

# Chapter Three: Materials and Methods

## 3.0 Introduction

This chapter describes materials and methods used in this study. It includes experimental design for development and optimisation of extraction conditions and analytical techniques used to characterise the olive extracts. Three extraction methods were proposed in this study, they were (i) conventional solvent extraction using water/ethanol-based solvent (ii) infusion using boiling water and (iii) ultrasonic assisted solvent extraction.

A multilevel factorial experiment design was carried out for the first method with the aims of determining optimum extraction conditions associated with the method.

Boiling water infusion was evaluated as water as a solvent is nontoxic and cheap. The extraction process is very quick and simple. Two different solvent: solid ratios were used for boiling water infusion in order to determine the impact of boiling water and ratio on the extraction yield.

To investigate the effect of ultrasound on extraction efficiency, ultrasound water bath was adopted and extraction was carried out under optimum conditions defined from method (i).

There were three key indicators used in this project for assessment of extract quality. They are total phenolic content determined spectrophotometrically using the Folin-Ciocalteu reagent, the oleuropein level determined by HPLC, antioxidant capacity determined by two in vitro assays. In this chapter, the protocol for measurement of total phenolic content and antioxidant capacity and the technique for determination of phenolic profile in extracts will be described.

## 3.1 Extraction of Phenolic Compounds from Olive Leaf

### 3.1.1 Leaf Materials and Reagents

Olive leaves (*Olea europaea* var. 'Frantoio') were collected from the Village Press in June 2010. The leaves were recovered from the factory after the de-leafer which is used to separate the leaves from the olives prior to processing. After collection, the leaves were transferred to the laboratory. The twigs were removed from leaves, and fresh leaves were

washed with tap water. The clean leaves were air dried at 20±2°C for 4-6 hours to remove surface moisture before continuing drying at 30 ±0.5°C in an incubator for 48 hours (Japon-Lujan & De Castro, 2006; Silva et al., 2006; Japon-Lujan & De Castro, 2008, Boudhrioua et al., 2009; Lee et al., 2010). Dried leaves were vacuum-packed in aluminium foil bags to avoid oxygen and light, and then stored in a freezer at -20±2°C until use.

Before each extraction, dried olive leaves were ground in a coffee grinder (Sunbeam EM0415), and sieved to ensure particles where ≤1mm particle size. Fresh and dried olive leaves were randomly sampled for moisture content determination.

Absolute ethanol was obtained from Polychem/Anchor Ethanol (Auckland, New Zealand); analytical grade methanol (Fisher Chemicals) was purchased from ThermoFisher Scientific (Auckland, New Zealand). Distilled water was used for preparing a solution of ethanol/water 80:20 (v/v), 70:30 (v/v) and 60:40 (v/v), and a solution of methanol/water 80:20 (v/v).

### 3.1.2 Stability of Dried Olive Leaf Samples

In order to ensure there were minimum changes and variability in total phenolcis as well as phenolic composition between the samples used for extraction, dried olive leaf samples were stored at -20±2°C. The stability of dried olive leaf samples was monitored. Control samples of 5 gram powdered dried olive leaves was extracted using 100ml 80% aqueous methanol (v/v). Extractions were carried out when the leaves were first dried then repeated every 4 days for 16 successive days until the end of extraction optimisation process. The powdered leaves were extracted in the 80% aqueous methanol with constant stirring (magnetic stirring at 400 rpm) in the dark at 20±2 °C for 24 hours. The extracts were filtered through Whatman No 2 qualitative filter paper and the flitered solution was then analysed for total phenolic content and oleuropein.

### 3.1.3 Extraction of Olive Leaf Phenolics Using Ethanol:Water Based Solvent Extraction Procedure

Approximately 5 g of powdered dried olive leaves were placed in 250 ml Schott glass bottles, the solvent consisting of varying amounts of aqueous ethanol was weighed and added into bottle according to different solvent: solid ratio (w/w). The extraction was carried out at the desired extraction temperature by using an OLS 200 shaking water bath (Grant, UK) for predetermined time periods; the shaking frequency was controlled at

80 - 100 times per minute. In order to avoid ester bond hydrolysis, the pH of the solvent was measured to ensure it was under pH 8 (Japon-Lujan & De Castro, 2006). When the extraction was completed, the extracts was filtered through Whatman No. 2 filter paper and stored at -80°C in 25ml amber glass bottle until analysis.

**Experimental Design**

A multilevel factorial experimental design as shown in Table 3.1 was used to identify the relationship between the response variable (extraction yield) and the process variables, as well as to determine the conditions that optimised the extraction process. The three independent factors studied were ethanol concentration which varied between 60, 70 and 80% (v/v), temperature varied between 20°C and 40°C and solvent: solid ratio (w/w) varied between 20 and 30.

In order to determine optimum extraction time, the extracts from treatment 1 and 12 were sampled every 12 hours, a total of eight samples were taken and total phenolics and oleuropein content of those samples were determined. The impact of extraction time on extraction yield was determined by plotting extraction yield (total phenolics content & oleuropein level) against extraction time. The time which gave the highest extraction yield was selected as standard extraction time for subsequent experiments. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model.

Table 3. 1: Experimental Design for Extraction Conditions

| Treatments | Temperature(°C) | Solvent:solid ratio(Wt:Wt) | Ethanol concentration (%) |
|---|---|---|---|
| 1 | 20 | 20 | 60 |
| 2 | 20 | 20 | 70 |
| 3 | 20 | 20 | 80 |
| 4 | 20 | 30 | 60 |
| 5 | 20 | 30 | 70 |
| 6 | 20 | 30 | 80 |
| 7 | 40 | 20 | 60 |
| 8 | 40 | 20 | 70 |
| 9 | 40 | 20 | 80 |
| 10 | 40 | 30 | 60 |
| 11 | 40 | 30 | 70 |
| 12 | 40 | 30 | 80 |

### 3.1.4 Extraction of Olive Leaf Phenolics Using Boiling Water

Approximately 5 g of powdered leaf sample was weighed and extracted with either 100ml or 150ml boiling water. During extraction the solution was boiled for a period of 10

minutes, and then the extract was cooled on the bench for 10 minutes and filtered with Whatman No. 2 filter paper (Malik & Bradford, 2008; Silva et al., 2006). The extract was then stored at -80°C in 25ml amber glass bottle until analysis.

### 3.1.5 Ultrasonic Assisted Solvent Extraction of Olive Leaf Phenolics

Dried and sieved olive leaf sample (500 mg) was extracted three times at 40±1.0°C with 80% aqueous ethanol (15ml +5ml+5ml). The extraction was performed in a 35 kHz Sonorex Super RK510 ultrasonic bath (Bandelin, Germany) and the duration of the first and subsequent steps was 30 and 15 minutes respectively. After each extraction step, the mixture was centrifuged at 3000 rpm for 5 minutes at 20°C in an IEC centra CL3R centrifuge (Thermo Scientific, New Zealand), and the supernatant was recovered and combined (Le Floch et al., 1998; Albu et al., 2004; Adam et al., 2009; Goulas, 2010;).

### 3.1.6 Determination of Dry Matter (DM) and Moisture Content

The dry matter (DM) was measured by determining the weight difference after drying the samples, following the AOAC method No.964.22 (AOAC, 1990). Fresh and dried olive leaves were cut to small pieces and weighed into a dry pre-weighed aluminum dish, and the dish was then placed in an air oven (Contherm, New Zealand) at 105 ± 0.5°C for 4 hours. After drying, the sample was cooled in desiccators for approximately 30 minutes. The DM content was calculated as shown in Equation 3.1. The moisture content was determined as 100 - %DM.

$$\%DM = \frac{(\text{Weight of sample after drying} + \text{Weight of moisture dish})}{(\text{Weight of sample before drying} + \text{Weight of moisture dish})} \times 100\% \qquad (3.1)$$

### 3.1.7 Determination of Total Content of Phenolic Compounds Using Folin–Ciocalteu Reagent

**Reagents**

The following reagents were used for the analyses of total phenolics: Folin-Ciocalteu reagent (2 M) and analytical grade caffeic acid were obtained from Sigma (Auckland, New Zealand); reagent grade sodium carbonate ($Na_2CO_3$) was from Scharlau (Barcelona, Spain). A stock solution of Folin-Ciocalteu reagent (0.2M) and sodium carbonate solution (75 g/L) was prepared with distilled water. Standard stock caffeic acid solution (0.5

mg/mL) was prepared in absolute methanol. This standard solution was stable for several weeks at -20°C.

**Determination of Total Phenolic Content of Olive Leaf Extract**

Phenolic content in olive leaf extract was determined according to the Folin–Ciocalteu method based on a colorimetric oxidation/reduction reaction (Singelton et al., 1999; Silva et al., 2006; Bahloul et al., 2009;).Caffeic acid was used as standard because it is one of phenolic components in olive leaf extract and more active than gallic acid in FC method (Stratil et al., 2006; Savournin et al., 2001). One milliliter of appropriately diluted extract sample was pipetted into 15 mL falcon tubes. A series of dilutions of standard caffeic acid solution (0.5 mg/mL) were prepared with absolute methanol. The Folin-Ciocalteu reagent (5 mL) was added into each sample or standard caffeic acid solution followed by thoroughly mixing with a vortex mixer. Sodium carbonate solution (4 mL) was then added no earlier than 30 seconds and not later than 8 minutes after addition of the Folin-Ciocalteu reagent. The capped tubes were incubated for 120 minutes in an incubator at 20±0.5°C and the absorbance was measured at 760 nm using a UV-1700 spectrophotometer (Shimadzu, Japan). The standard curve was prepared using 0.0125, 0.025, 0.05, 0.075 and 1.0 mg/ml solutions of caffeic acid in absolute methanol.

The total phenolic content was calculated using Equation 3.2 for the linear standard curve obtained from plotting the absorbance values (at 760 nm) against concentration of caffeic acid.

$$\text{Absorbance of standard caffeic acid} = \text{Slope} \times \text{Concentration of standard caffeic acid} + \text{Intercept} \quad (3.2)$$

The results were reported as caffeic acid equivalent (CAE) in milligram of caffeic acid per kilogram of dry leaves (mg caffeic acid $kg^{-1}$dry matter) or milligram of caffeic acid per gram of olive leaf extract (mg caffeic acid $g^{-1}$ olive leaf extract) .

$$\text{The total phenolic content (mg/kg dry matter)} = \frac{(\text{Absorbance of Leaf extract sample} - \text{Intercept}) \times \text{Dilution factor} \times 1000}{\text{Slope} \times \text{Weight of dry matter}} \quad (3.3)$$

$$\text{The total phenolic content (mg/g olive leaf extract)} = \frac{(\text{Absorbance of Leaf extract sample} - \text{Intercept}) \times \text{Dilution factor}}{\text{Slope} \times \text{Weight of olive leaf extract}} \quad (3.4)$$

## 3.2 High-Performance Liquid Chromatography (HPLC) Analysis for the Quantitative Determination Of Phenolic Profile Of Olive Leaf Extract

### 3.2.1 Reagents

Analytical grade glacial acetic acid was provided by Biolab Pty Ltd (Clayton, Australia). HPLC grade acetonitrile was from Merck (Darmstadt, Germany). The solvent used for HPLC mobile phase was prepared in distilled water, and then filtered through 47 mm/0.22µm (diameter/pore size) Millipore Nylon membrane filters (Massachusetts , USA) and degassed in an ultrasonic bath before use.

### 3.2.2 Standards

Oleuropein, verbascoside, hydroxytyrosol, apigenin-7-O-glucoside, luteolin-7-O-glucoside, luteolin-4-O-glucoside luteolin, quercetin-3-O-glucoside and rutin were obtained from Extrasynthese (Genay, France). Vanillic acid, vanillin, tyrosol, ferulic acid, caffeic acid, quercetin dehydrate, apigenin, p-courmaric acid and o-courmaric acid were from Sigma (Auckland, New Zealand). All standards were HPLC grade except vanillin, caffeic acid, quercetin dehydrate and p-courmaric acid, which were analytical grade.

Table 3. 2: Purity Specification, Grade and Brand for Standards

| **Compound name % purity specification** | **Grade, brand** |
|---|---|
| Gentistic acid ≥98% (2,5-dihydroxybenzoic acid) | HPLC, Aldrich |
| Benzoic acid ≥99.5% | ACS reagent, Sigma-Aldrich |
| Vanillic acid (4-hydroxy-3-methoxybenzoic acid) ≥97% | HPLC, Fluka |
| Vanillin(4-hydroxy-3-methoxybenzaldehyde) | HPLC, Fluka |
| Protocatechuic acid ≥97% (3,4-dihydroxybenzoic acid) | Purum, Aldrich |
| p-hydroxybenzoic acid ≥99% | HPLC, Aldrich |
| Syringic acid ≥95% | HPLC, Sigma |
| Caffeic acid | Analytical, Sigma |
| p-coumaric acid (4-hydroxycinnamic acid) | HPLC, Sigma |
| o-coumaric acid ≥97% ( trans 2-hydroxycinnamic acid) | HPLC, Sigma |
| Ferulic acid ≥99% | HPLC, Aldrich |
| Sinapinic acid ≥99% | HPLC, Aldrich |
| (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA) ≥97% | HPLC, Sigma-Aldrich |
| p-hydroxyphenylethanol (p-HPEA) or tyrosol ≥90% | HPLC, Sigma-Aldrich |
| Verbascoside | HPLC, Extrasynthese Genay France |
| Oleuropein ≥80% | HPLC, Extrasynthese Genay France |
| Apigenin ≥95% | HPLC, Sigma-Aldrich |
| Luteolin ≥99% | HPLC, Extrasynthese Genay France |
| Quercetin-3-O-glucoside ≥99% | HPLC, Extrasynthese Genay France |
| Rutin ≥99% | HPLC, Extrasynthese Genay France |
| Luteolin-7-O-glucoside ≥98% | HPLC, Extrasynthese Genay France |
| Quercetin dehydrate ≥98% | HPLC, Extrasynthese Genay France |
| Luteolin-4-O-glucoside ≥95% | HPLC, Extrasynthese Genay France |

### 3.2.3 Analytical Technique and Equipment

The HPLC equipment used was a VP series HPLC system (Shimadzu, Japan) equipped with SCL-10A system controller, a photodiode array detector (SPD-M10AVP). The stationary phase was a C18 Synergi Fusion RP 80 column (100×4 mm i.d.) with a particle size of 3.5 µm (Phenomenex, USA), the temperatures of the column was controlled at 30°C. The flow rate was 0.8 ml/min and the absorbance changes were monitored at 245nm, 280 nm, 330nm, 340nm and 350nm. The mobile phases for chromatographic analysis were: (A) acetic acid/water (2.5:97.5) and (B) acetonitrile. A linear gradient was run according to method of Benevento et al., (2000) as shown in Table 3.3; a few modifications were made in order to reduce total run time to 45 minutes.

Table 3. 3: HPLC Gradient for Separation of Phenolics Compounds on Synergi Fusion RP 80 Column (Benevento et al., 2000)

| **Time(minutes)** | **Solvent A (%)**<br>**Water/acetic acid(97.5:2.5 v/v)** | **Solvent B (%)**<br>**Acetonitrile** |
|---|---|---|
| **0** | 95 | 5 |
| **20** | 75 | 25 |
| **15** | 50 | 50 |
| **5** | 20 | 80 |
| **5** | 95 | 5 |

The data was processed on "LC solution" software. Phenolic compounds in olive leaf extract were identified by comparison of their retention times with the corresponding standard and by their UV spectra obtained with the diode array detector. Quantitative analysis was performed with calibration curves obtained using pure standards. The external standard method was employed, three-point calibration curves were used, and the regression coefficients were in the 0.980-1.00 range.

All olive leaf extract samples were filtered through 0.45µm syringe filter (Raylab, NZ) with a 25ml Luer lock syringes (Thermofisher Scientific, NZ) prior to HPLC determination and 20µl of samples were injected into HPLC. Phenolic compounds in olive leaf were expressed in milligram of phenolics identified per kilogram of dry leaves (mg (kg dry matter)$^{-1}$). Phenolic compounds in olive leaf extract were in milligram of phenolics per gram of extract (mg g$^{-1}$).

For example, Oleuropein concentration was determined using pure oleuropein standards. The peak area of pure standards at three different concentrations in chromatogram were plotted against the concentrations of pure standards to obtain a calibration curve, then concentration of oleuropein in olive leaf extracts was calculated based on the slope and intercept of calibration curve.

## 3.3 Determination of Extraction Efficiency of Methods

Methanol was reported as the best solvent for extraction of phenolic compounds from olive leaf as mentioned in literature review. The extraction efficiency of three proposed extraction methods was determined by comparing extraction yield (total phenolics & oleuropein) of each method with "maximum extraction yield", which was obtained by extracting the same olive leaf samples using 80% aqueous methanol (v/ v) as a solvent under optimum extraction condition defined in the study, which was the temperature of 40±1°C and the solvent to solid ratio of 30. Extraction efficiency was calculated as a percentage of yields of each extraction method with respect to maximum yield.

## 3.4 The Effect of Cultivar, Harvest Time and Drying On Phenolic Compounds of Olive Leaf Extract

Two cultivars of fresh olive leaf (*Olea europaea* var. 'Frantoio' and 'Barnea') were provided by The Village Press in June, October and November, 2010. The fresh leaves were ground immediately and approximately 5 gram of fresh leaves was extracted using 100 ml 80% methanol at 40±2 $^{0}$C for 24 hours with agitation in the dark.

Olive leaves from the two cultivars was dried in an incubator at 30±0.5 °C for 48 hours. Then dried leaves were ground and extracted using the same condition as fresh leaves. . All the extracts were filtered through Whatman No.2 filter paper, and total phenolic content and phenolic composition of each extract was measured using same methods described previously in section 3.1.7 & section 3.2.

## 3.5 The Effect of Drying Methods on the Extraction Yield

Two drying methods have been investigated in order to identify the effect of different drying methods on extraction yield. Fresh olive leaves (*Olea europaea* var. 'Frantoio') were dried under two conditions: (i) in an incubator at 30±0.5$^{0}$C for 48 hours and (ii) in a microwave oven for 2 minutes at maximum power (1000W) (Bouaziz & Sayadi, 2005;

Savournin, 2001). Five grams of dried and grinded olive leaves were extracted using 150 ml 80% aqueous ethanol at 40±0.5 $^{0}$C in a shaking water bath for 24 hours respectively, and the extracts were filtered through Whatman No.2 filter paper and analysed for total phenolic content and phenolic composition by HPLC.

## 3.6 Evaluation of Antioxidant Capacity of Olive Leaf Extract

### 3.6.1 Reagents

2, 2-diphenyl-1-picryhydrazyl radical (DPPH), (±)-6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescent sodium salt were obtained from Sigma-Aldrich. 2,2'-azobis-2-methyl-propanimidamide,dihydrochloride (AAPH) was purchased from Cayman Chemical (Florida, U.S.A). All chemicals used were of analytical grade.

### 3.6.2 DPPH$^{\cdot}$ Radical Scavenging Activity Assay

Antioxidant capacity of olive leaf extract was measured using the DPPH assay as described by Bouaziz et al., (2004). Four milliliters of approriately diluted olive leaf sample and 10ml of the DPPH (1.5 x10$^{-4}$ M) working solution were gently mixed in a glass tube and left in an incubator at 20±0.5°C for 40 minutes. Incubation time of 40 minutes was determined experimentally and considered to be sufficient for this study.

Oleuropein solution 40 µg/mL in methanol (4 mL) was added to 10 mL of 1.5 x10$^{-4}$ M methanol DPPH$^{\cdot}$ radical solution. After gently mixing two solutions they were incubated at 20°C, the decrease in absorbance was monitored at 520 nm at 0min, 3 min and every 5 min until the reaction reached a plateau. The result showed that 40 minutes of incubation time was sufficient (Figure 3.1).

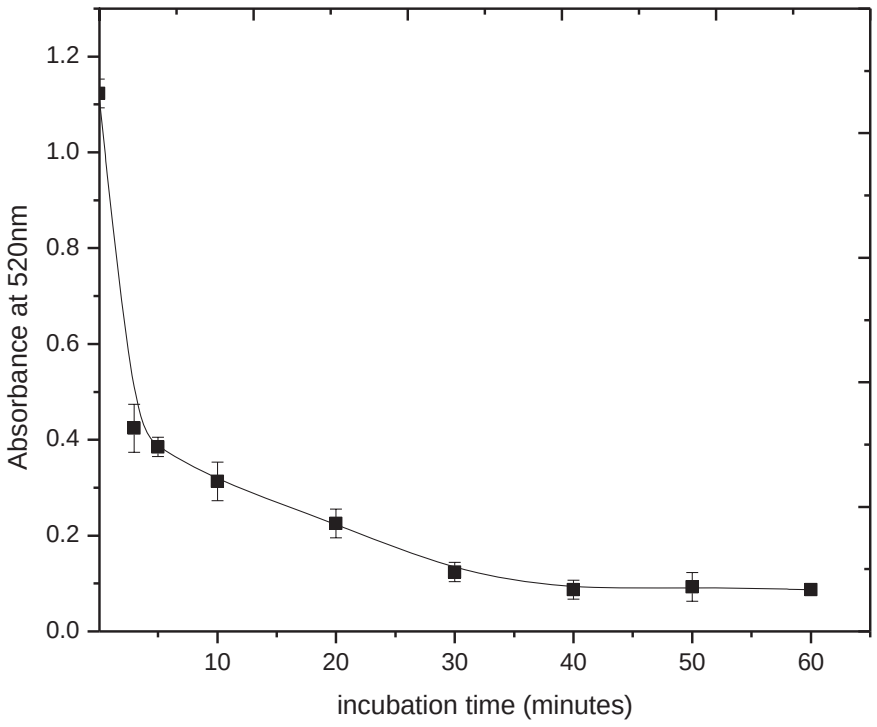



Figure 3. 1: Variation of the DPPH Absorbance with Time in The Presence Of Oleuropein

The absorbance was measured against methanol (control) at 520 nm using a UV-1700 spectrophotometer (Shimadzu, Japan). The percentage of DPPH radical scavenging activity relative to the control was calculated using the following Equation 3.5:

$$\%\text{Radical scavenging activity} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{\text{Absorbance of Control}} \text{X100\%} \quad (3.5)$$

Then the percentage of DPPH radical scavenging activity was plotted against the olive leaf extract concentration (mg/ mL) to construct a log-dose inhibition curve. The antioxidant activity of each test sample was expressed in terms of concentration required to inhibit 50% DPPH radical formation (called EC50, in mg/mL) and calculated from the log-dose inhibition curve. A protocol of DPPH assay used in the study is given in the Appendix 3.1.

### 3.6.3 Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was performed on a FLUOstar Optima automated micro plate reader (BMG Labtech, Germany) with 96-well plates with fluorescence filters for an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Antioxidant capacity of olive leaf extract was measured by inhibition of the peroxyl-radical induced oxidation initiated by thermal decomposition of a biological relevant radical source, AAPH, as described in the method by Ou et al.(2001) and Huang et al. (2002 ). Briefly, to each well 25µl appropriately diluted olive leaf extract samples or 75 mM phosphate buffer (pH 7.4) (blank) or standard (Trolox) were added to 150 µl of working sodium fluorescein solution. Reactions were initiated by the addition of 25 µl of 153 mM AAPH reagent. Measurement temperature was set at 37 °C. The intensity of fluorescence was monitored kinetically with data taken every minute for a total of 70 minutes (Figure 3.2).

ORAC values were calculated as described by Prior et al.(2005). The fluorescence (FL) decay curve was generated by plotting fluorescence intensity of blank or sample or standard against time. The area under FL curve (AUC) and the Net AUC of the standards ($AUC_{standard}$ - $AUC_{blank}$) and olive leaf extract samples ($AUC_{sample}$ - $AUC_{blank}$) were determined using Equations 3.6.

$$AUC = 0.5 + f_1/f_0 + \ldots f_i/f_0 \ldots + f_{59}/f_0 + f_{70}/f_0 \qquad (3.6)$$

Where $f_0$, $f_i$, $f_{70}$ are the fluorescent readings at 0 minutes, i minutes and 70 minutes

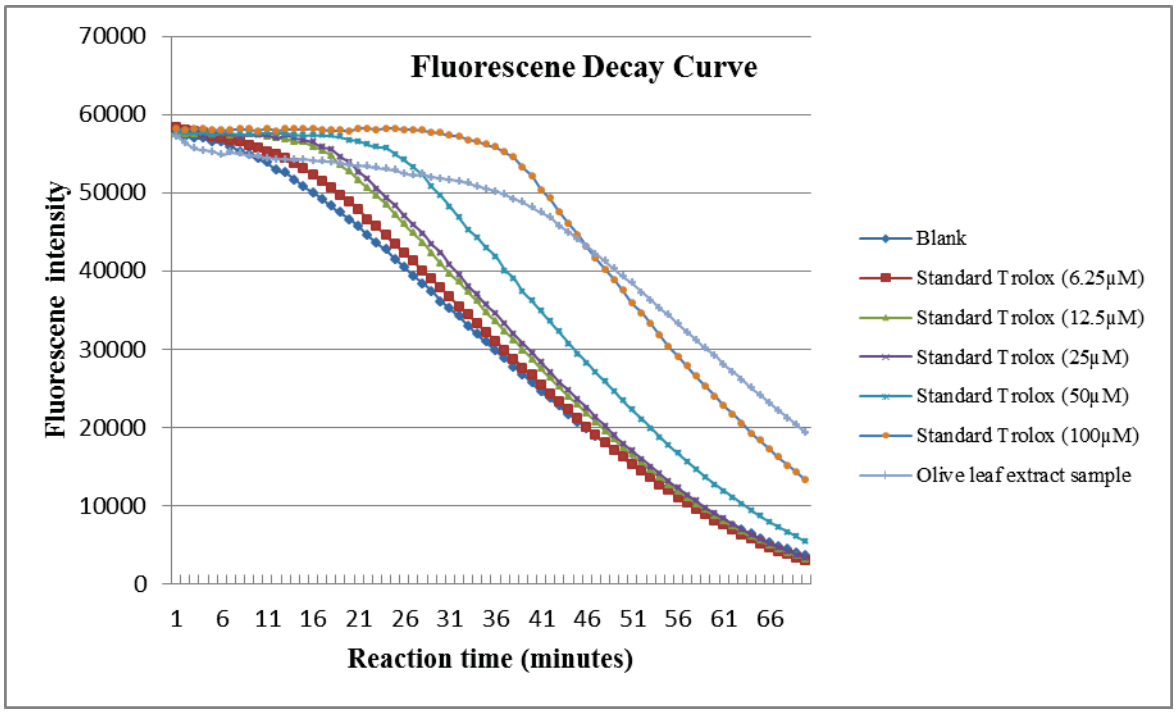



Figure 3. 2: Example of a Fluorescence Decay Curve for the ORAC Method

Ranges of 6.25-50 µM Trolox dilution were made from stock standard solution (0.02M) in 75mM phosphate buffer (pH 7.4). A standard curve was generated using the Net AUC for five standard concentrations of Trolox, and the linear relationship (Equation 3.7) was obtained between the Net AUC and concentration of standard Trolox. ORAC value of olive leaf extract was calculated from Equation 3.8 expressed as micromoles of Trolox equivalents (TE) per gram of extract (µmol of TE $g^{-1}$). A protocol of ORAC assay and an example of detail calculation is given in Appendix 3.2.

$$\text{Net AUC of standard Trolox} = \text{Slope} \times \text{Concentration of standard Trolox} + \text{Intercept} \qquad (3.7)$$

$$\text{Trolox equivalents (µmol of TE/g olive leaf extract)} = \frac{(\text{Net AUC of Leaf extract sample} - \text{Intercept}) \times \text{Dilution factor}}{\text{Slope} \times \text{Weight of olive leaf extract}} \qquad (3.8)$$

## 3.7 Investigation of Stability of Olive Leaf Extract

### 3.7.1 Preparation of Olive Leaf Extract

Two batches of olive leaf extract was prepared as follows: 5 gram of dried, grinded olive leaf (*Olea europaea* var. 'Frantoio') was weighed in a 250ml Schott bottle and extracted using 150ml of 60% or 80% aqueous ethanol (v/v) at 40±2°C in a shaking water bath in the dark for 24 hours. Two different concentrations were used in order to identify the impact of ethanol concentration on degradation of phenolic compounds during storage.

A total of 60 gram of dried olive leaves in 12 bottles was used for each batch. After extraction was completed, the extracts were filtered through Whatman No.2 filter paper and then the aqueous phase were evaporated to10 fold concentration (by volume ) using a RE111 rotary evaporator (Buchi, Germany) at a mild temperature of 40-45°C and vacuum pressure of 758 kPa.

### 3.7.2 Storage Conditions

For each sample, 5 to 10 ml of concentrated olive leaf extract was placed into a 25 ml amber glass bottle, and then topped with nitrogen and sealed. The storage temperatures and time of extracts were -20±2°C for 10 weeks, 4±1°C for 10 weeks, and 20±0.5°C for 12 weeks and 32±1°C for 12 weeks. The total phenolic content and oleuropein concentration of each extract were monitored every week for the first 6 weeks, then every alternate week for the rest of the storage period. Antioxidant capacity of leaf extracts were also measured every two weeks during storage. Concentrated olive leaf extract was reconstituted with 60% or 80% aqueous ethanol before each measurement.

### 3.7.3 Evaluation of Microbiological Safety of Olive Leaf Extract

To determine the microbiological safety of the olive leaf extract, standard plate counts was carried out at the beginning, in the middle and at the end of the storage period. Standard plate count was performed according to the method of Larry & James (1998). The dilutions of $10^{-1}$ and $10^{-2}$ were prepared by transferring 1ml of previous dilution to 9ml peptone water. The steps followed were :Pipette 1 ml of each dilution into separate, duplicate, appropriately marked Petri dishes, then pour 12-15 ml plate count agar to each plate, immediately mix sample dilutions and agar medium thoroughly , and incubate promptly for 48 ± 2 h at 35°C after agar solidifies. The result is expressed as CFU (colony forming unit)/ml.

## 3.8 Determination of Oleuropein Concentration of Commercial Olive Leaf Products

Two commercial olive leaf extract products were tested. For 'Oliviral' olive leaf extract, powder in one capsule was dissolved in 100 ml 80% ethanol (v/v). For 'Comvita" olive leaf extract, 1 ml products was diluted with 50 ml 80% ethanol (v/v). Then oleuropein in both solutions was measured using the method descried in Section 3.3.

## 3.9 Statistical Analysis

The extraction experiments in all sections above were carried out in triplicates. All determinations were also carried out at least in triplicate, mean values were determined and the standard error of the mean (±SEM). Linear regression analyses were performed using Microsoft Excel 2007. Correlations among data obtained were calculated using Pearson's correlation coefficient. Analysis of variance (ANOVA) and two samples T test was carried out using Mintab 15 (Minitab Inc, USA). Differences at $P < 0.05$ were considered statistically significant.

# Chapter Four: Results and Discussions

## 4.1 Stability of Dried Olive Leaf

In order to ensure there was minimum change or variability between leaf samples (var. 'Frantoio') used for extraction experiments and to avoid degradation of phenolics compounds, dried olive leaf samples were stored at -20°C, total phenolics content and oleuropein concentration of the control samples was monitored every 4 days. Figures 4.1.a & 4.1.b show the changes of both values of the control sample during the extraction experiments, respectively. The total phenolic content remained constant, whereas, oleuropein levels decreased, but not significantly ($P \geq 0.05$) after 12 days storage. The result suggests that dried olive leave are relatively stable when stored at -20 °C, there was no significant degradation of total phenolics and oleuropein concentration($P \geq 0.05$) occurring during experimental period. Oleuropein is prone to hydrolysis, it breaks down to hydroxytrosol, oleuropein aglycone (Mourtzinos et al.,2007), which are still detected in the total phenolic assay hence no variations observed in this value, but oleuropein values do decrease hence it was monitored prior to the extraction process.

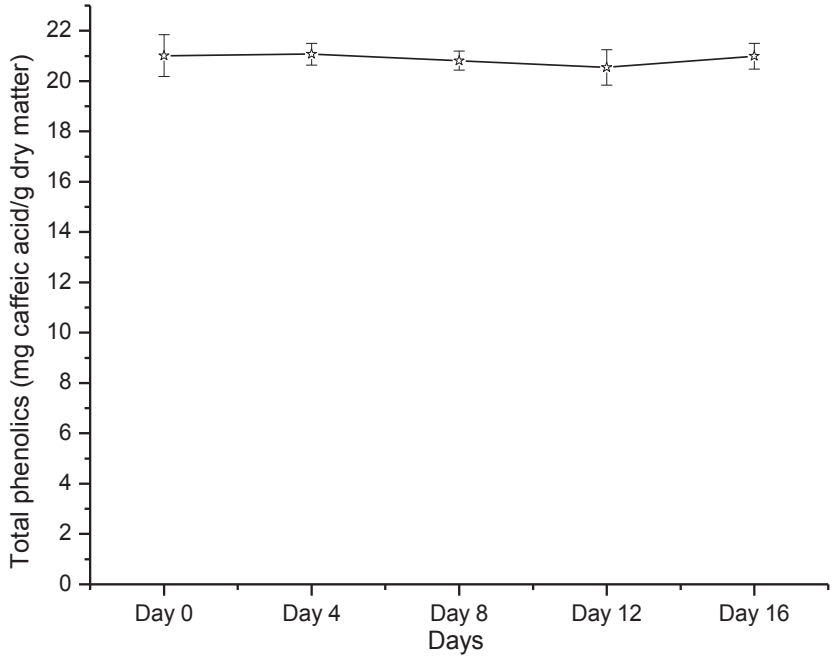



Figure 4. 1.a: Variations of Total Phenolic Content of Dried Olive Leave with Storage Time When Stored At -20±2°C. All Experiments Were Carried Out in Triplicates and Results Are Expressed As Mean ±SEM (N=3)

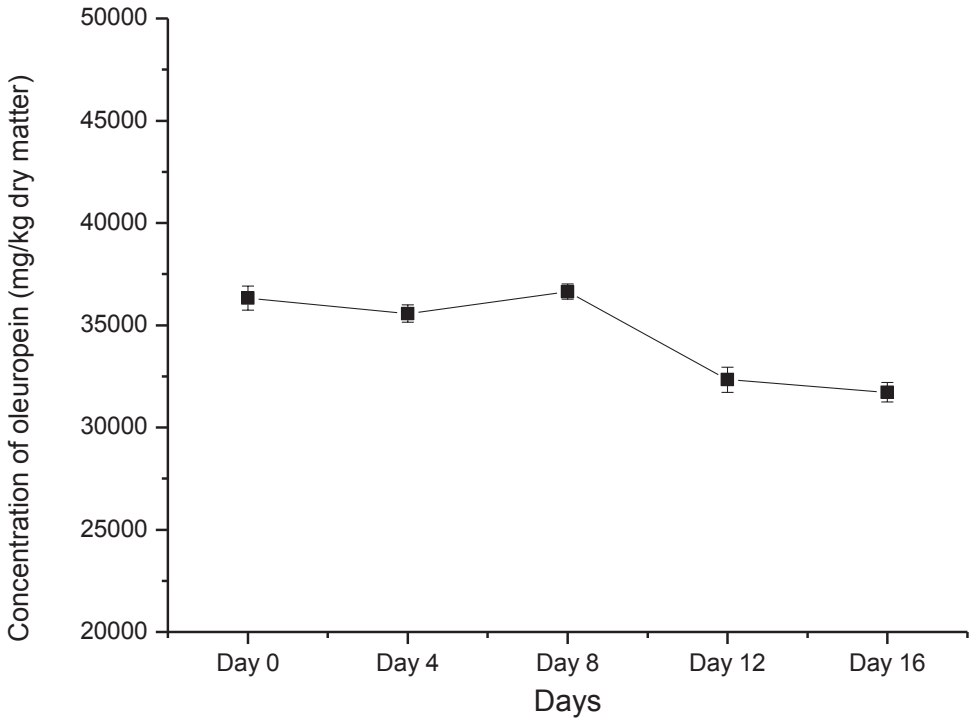



Figure 4.1.b: Variations of Oleuropein of Dried Olive Leave with Storage Time When Stored At -20±2°C. All Experiments Were Carried Out in Triplicates and Results Are Expressed As Mean ±SEM (N=3)

## 4.2 Extraction of Olive Leaf Phenolics Using Water/Ethanol-Based Solvent

### 4.2.1 The Effect of Extraction Time on Extraction Yield

Figures 4.2.a & 4.2.b shows the effect of extraction time on total phenolics content and oleuropein obtained in olive leaf extracts. Two extreme treatment conditions were tested in order to determine the optimum extraction time: Treatment 1 (low extreme) was carried out using 60% aqueous ethanol (v/v) at a solvent to solid ratio of 20 (w/w) and temperature of 20±0.5°C, and Treatment 12 (high extreme) was using 80% aqueous ethanol (v/v) at a solvent/ solid ratio of 30 (w/w) and temperature of 40±1°C. Significantly higher level of total phenolic content ($P \leq 0.05$) was observed after 24 hours duration for both treatments. With regard to oleuropein concentration, there was no significant difference ($P \geq 0.05$) when extracting olive leaves at 20 ±0.5°C for a period 12, 24, 36 and 48 hours. However, when extracting olive leaves at 40±1°C, there was an initial increase in oleuropein from 12 hours to 24 hours, and then followed by a decrease after 36 hours extraction. Significantly higher level of oleuropein ($P \leq 0.05$) was recovered after 24 hours extraction at 40°C. It indicates

24 hours was sufficient for extraction of phenolics compounds from olive leaves, longer extraction time did not improve extraction yield. Hence, an extraction time of 24 hours were selected for further experiments.

When comparing total phenolic and oleuropein obtained by two treatments, it can be seen that significantly higher value of oleuropein and total phenolic compounds ($P \leq 0.05$) were found in the leaf extract obtained from treatment 12 (80% ethanol (v/v), solvent: solid of 30:1, temperature of 40°C). It gives an indication that higher level of phenolic compounds may be recovered with increasing ethanol concentration, temperature and solvent to solid ratio.

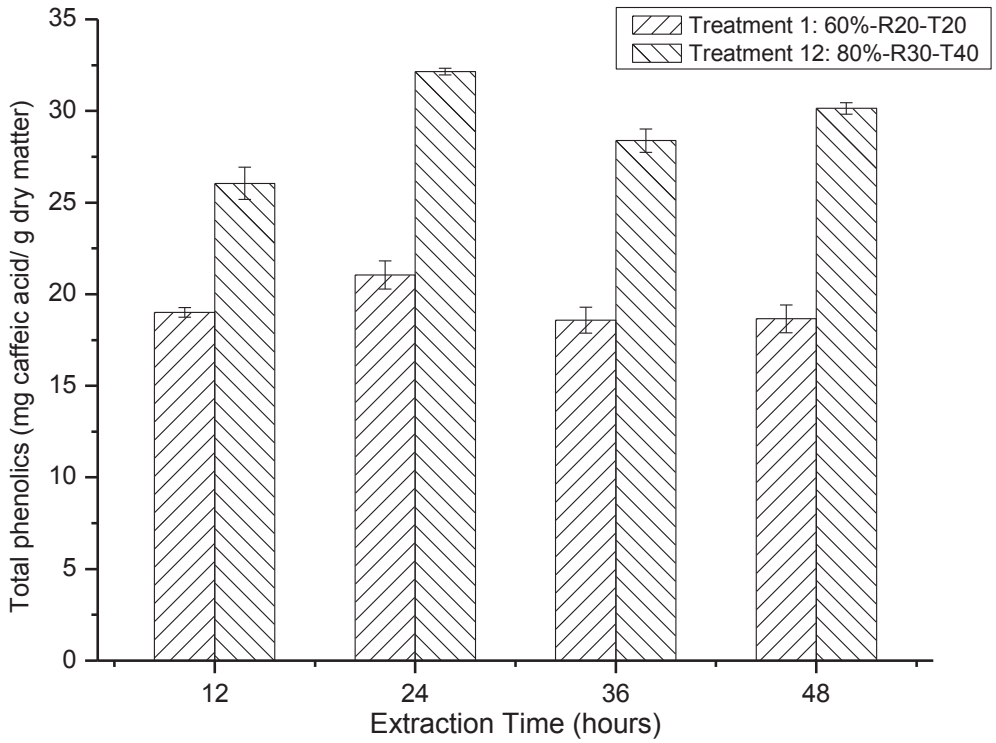



Figure 4. 2. a: The Changes of Total Phenolic Content in Olive Leaf Extracts Vs. Extraction Time. All Experiments Were Carried Out in Triplicates And Results Are Expressed As Mean ±SEM (N=3)

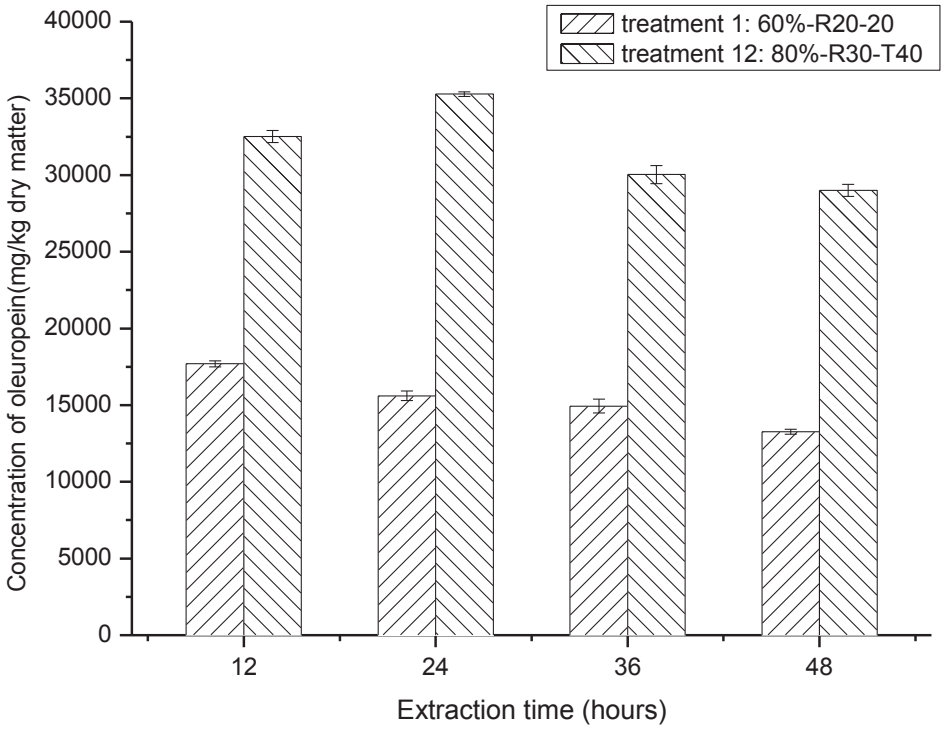



Figure 4. 2. b: The Changes of Oleuropein Concentration in Olive Leaf Extracts Vs. Extraction Time. All Experiments Were Carried Out in Triplicates and Results Are Expressed As Mean ±SEM (N=3)

### 4.2.2 Optimisation of Extraction Conditions

Three variables were considered for optimisation of the extraction step, namely: extraction temperature, ethanol concentration, solvent: solid ratio. The upper and lower values given to each variable were selected from the available data in the literatures. The response variables were the extraction yield expressed as the total phenolics and oleuropein concentration. The experimental design was shown in Table 3.1.

The total phenolics extracted were found to be between 19 to 26 mg caffeic acid equivalents/gram dry matters for all extracts obtained for 12 different treatments (Figure 4.3.a). This is in agreement with previous findings, which reported total phenolic content of dried olive was from 9.0 mg to 51.4 mg caffeic acid equivalent per gram dry matter (Boudhrioua et al., 2009). Relatively higher amount of phenolic compounds was observed in the extracts from treatment 10 (60% ethanol, solvent: solid ratio of 30:1, temperature of 40°C) and treatment 12 (80% ethanol, solvent: solid ratio of 30:1, temperature of 40°C). The oleuropein levels varied from 16,220 to 41,013 mg /kg dry matter and the highest

oleuropein was obtained using 80% ethanol at a solvent: solid ratio of 30 and a temperature of 40°C (Figure 4.3.b). The range of oleuropein was between 23,000 and 43,200mg mg/kg reported previously (Japon-Lujan et al., 2006; Jemai et al., 2008).

Figures 4.3.a & 4.3.b illustrate the combined effect of three variables on total phenolics and oleuropein recovery. It can be seen that increasing the temperature from 20°C to 40 °C produced a significant increase in both total phenolics content ($P \leq 0.05$) and oleuropein ($P \leq 0.05$) irrespective of the solvent: solid ratio and ethanol concentration. Regarding the effect of solvent: solid ratio, it can be seen that increasing from 20 to 30 produced a significant increase in both total phenolics and oleuropein level ($P \leq 0.05$). However, increasing ethanol concentration from 60% to 80% only produced a significant increase in oleuropein concentration ($P \leq 0.05$), the impact on the total phenolic content was not significant ($P \geq 0.05$). Overall, optimum extraction condition in this study was found to be: 40°C for extraction temperature; 30:1 for solvent: solid ratio (w/w); 24 hours for extraction time; 80% for ethanol concentration.

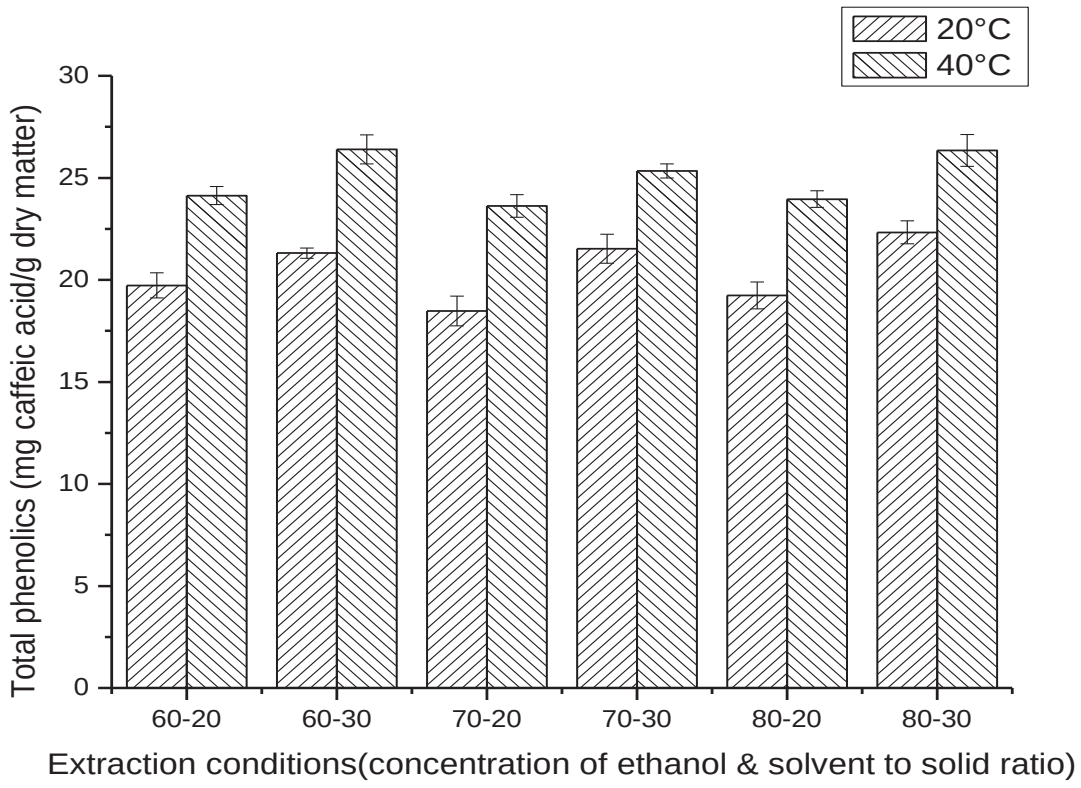



Figure 4. 3.a: Comparison of The Total Phenolics Extracted from Olive Leaf from 12 Different Treatments Given in Experiment Design, All Experiments Were Carried Out in Triplicates And Results Are Expressed As Mean ±SEM (N=3)

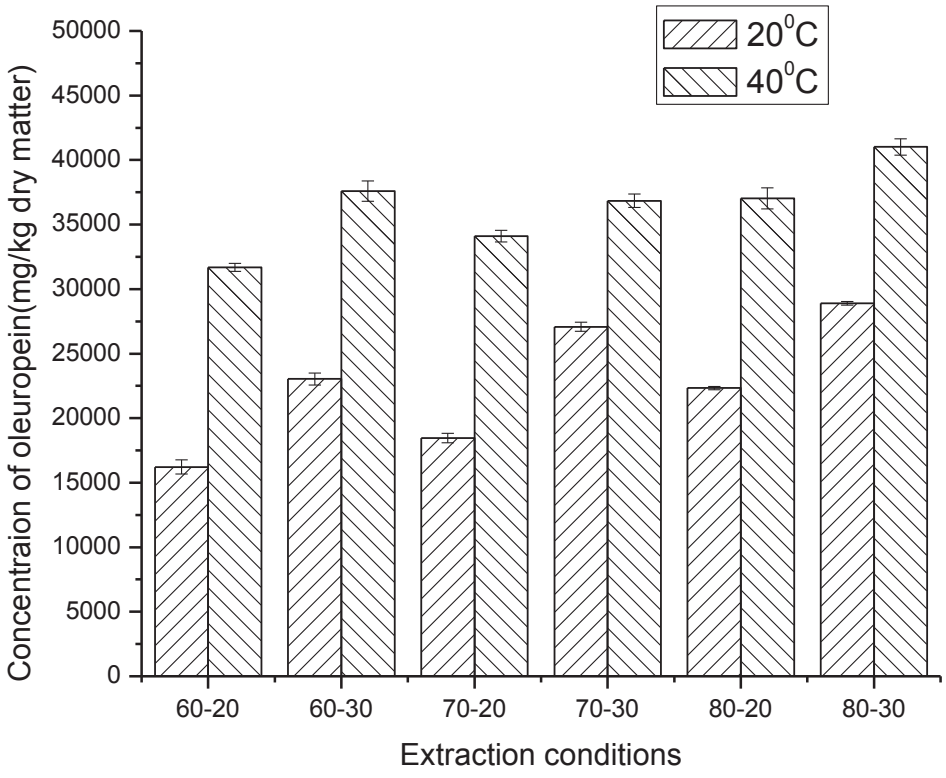



Figure 4. 3.b: Comparison of Oleuropein Concentration Extracted from Olive Leaf from 12 Different Treatments Given in Experiment Design, All Experiments Were Carried Out in Triplicates And Results Are Expressed As Mean ±SEM (N=3)

Optimum extraction condition may vary in different studies depending on the extraction parameters of interest and experimental design used in the study. Japon et al., (2006) reported the optimum working conditions for superheated liquid extraction of oleuropein and bioactive compounds from olive leave was: 70% ethanol (v/v) at 140°C for 13 minutes, for dynamic ultrasound-assisted extraction was: 60% ethanol (v/v) at 40°C for 25 minutes, for microwave assisted extraction was: 80% ethanol (v/v) for 8 minutes. The solvent: solid ratio used for all three studies was 8:1(v/v). Mylonaki et al. (2008) reported the optimum working condition for conventional extraction of olive leaf phenolic compounds was: 60% ethanol (v/v) with pH 2 for 5 hours, the temperature and solvent: solid ratio used in the study was 22±2°C and 40:1 respectively. Surface response methodology was used in these studies (Japon-Lujan et al., 2006; Mylonaki et al., 2008).

### 4.2.3 The Effect of Extraction Temperature on Extraction Yield

The impact of two extraction temperature (20°C, 40°C) on extraction yield was investigated. These temperatures were frequently used by researchers in previous studies (Bouaziz & Sayadi, 2005; Jemai, 2008; Japon et al., 2006; Kiritsakis et al., 2010). Analysis of variance (ANOVA) for total phenolics and oleuropein concentration (see Appendices 4.1 & 4.2) indicates that both values were significantly affected by temperature ($P \leq 0.05$). Figures 4.4.a and 4.4.b show the effect of temperature on total phenolics and oleuropein extracted from olive leaves, which were obtained by plotting the mean values of total phenolic and oleuropein for a total of 36 extractions against two different temperatures. As can be seen in the graph, total phenolics and oleuropein extracted at 40°C are significantly higher than 20°C ($P \leq 0.05$).

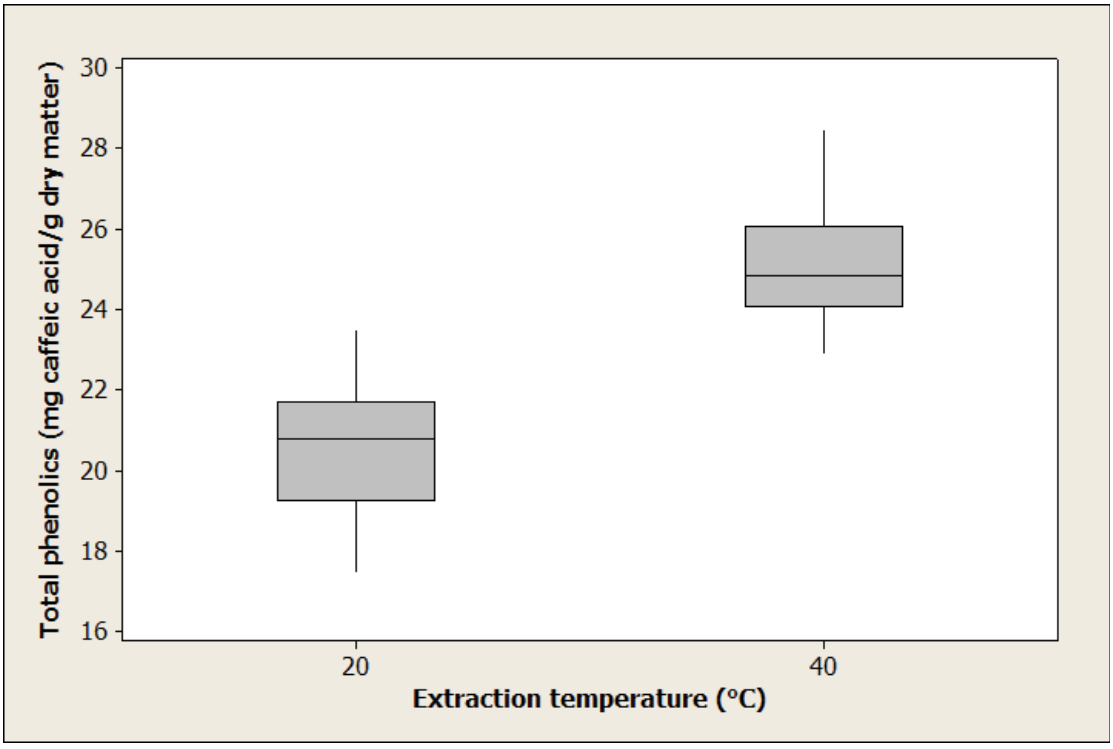



Figure 4. 4.a: Boxplot of Total Phenolics vs. Extraction Temperatures, All Experiments Were Carried Out in Triplicates And Data Points Are Mean Value of Triplicates (N=3)

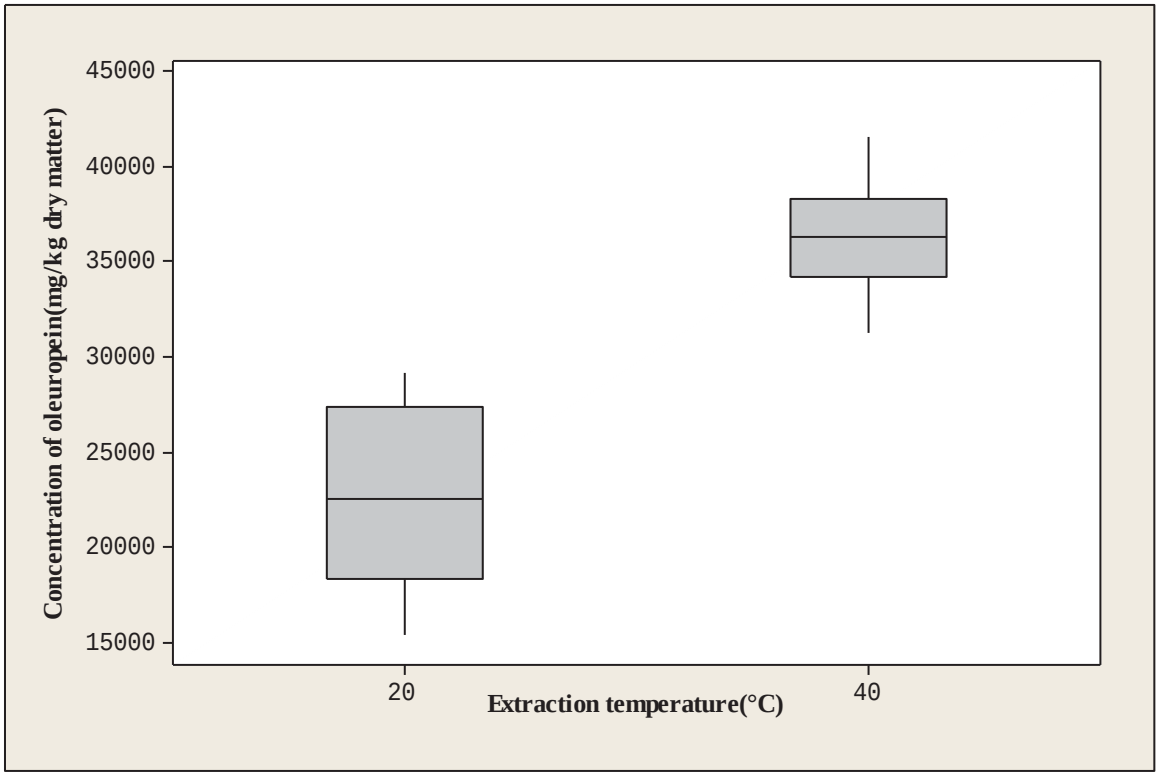



Figure 4.4.b: Box Plot of Oleuropein Concentration vs. Extraction Temperature, All Experiments Were Carried Out in Triplicates And Data Points Are Mean Value of Triplicates (N=3)

Increasing temperature favoured extraction by enhancing both the solubility of solute and the diffusion coefficient from a solid matrix to a liquid matrix as mentioned earlier in the literature review (Luthria, 2008). As a consequence, much higher values of the total phenolics content and oleuropein were observed at 40°C than 20°C. The results confirm that extraction temperature greatly influences the extraction yield ($P \leq 0.05$). Similar results about the effect of temperature on extraction of phenolic compounds were also reported for olive leaves by Japon et al. (2006), who found a relationship between temperature and extraction yields. At 40°C and for extraction times of 24 hours the amounts of bioactive olive phenolics compounds extracted were 95% , while at 30°C the amounts of bioactive olive phenolics compounds extracted were 75% for 24 hours, respectively (Japon-Lujan et al.,2006). Despite the fact of increasing temperature has the positive effect on the extraction yields, increasing temperatures also affect the stability of phenolic compounds (Malik et al., 2008). When extracting phenolic compounds from olive leaves at higher temperature, both effects need to be considered.

### 4.2.4 The Effect of Solvent: Solid Ratio on Extraction Yield

An increase of the solvent: solid ratio increased the concentration gradient and thus the rate of diffusion of the compounds from the solid to the solvent, leading to an increased extraction yield (Cacace & Mazza 2003). However, there is very little information available on the effect of solvent: solid ratio on the total phenolics extracted from olive leaves. In this work, two different solvent: solid ratios were investigated. Analysis of variance (ANOVA) for total phenolics and oleuropein concentration (see Appendices 4.1&4.2) indicates that the total phenolics and oleuropein concentrations are significantly affected by solvent: solid ratio ($P \leqslant 0.05$). The distribution of mean values of total phenolic and oleuropein for a total of 36 extraction is shown in Figure 4.5.a & 4.5.b, the higher the solvent to the solid ratio, the higher the total phenolics ($P \leq 0.05$) and oleuropein ($P \leq 0.05$) obtained, suggesting that the solvent:solid ratio also has a positive effect on the extraction yield. This is consistent with mass transfer principles; the driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater, when, a higher solvent: solid ratio is used (Pinelo et al., 2005).

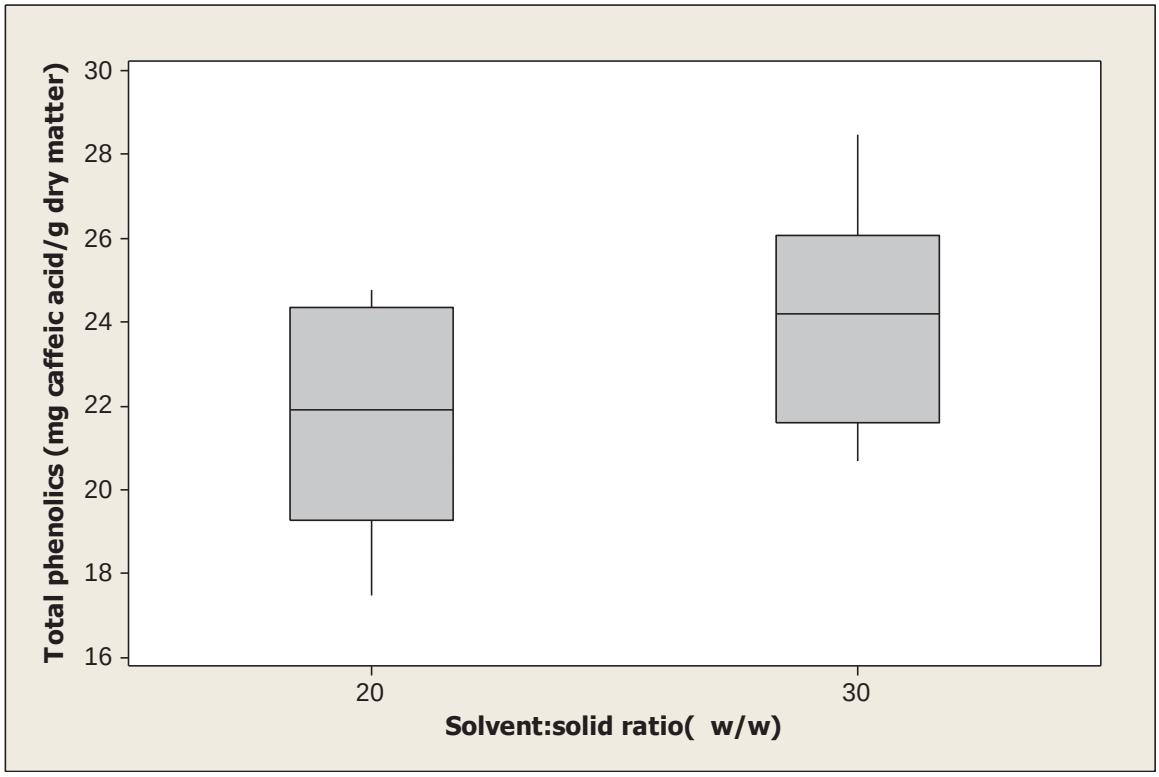



Figure 4. 5.a: Boxplot of Total Phenolics vs. Solvent: Solid Ratio, All Experiments Were Carried out in Triplicates and Data Points Are Mean Value of Triplicates (N=3)

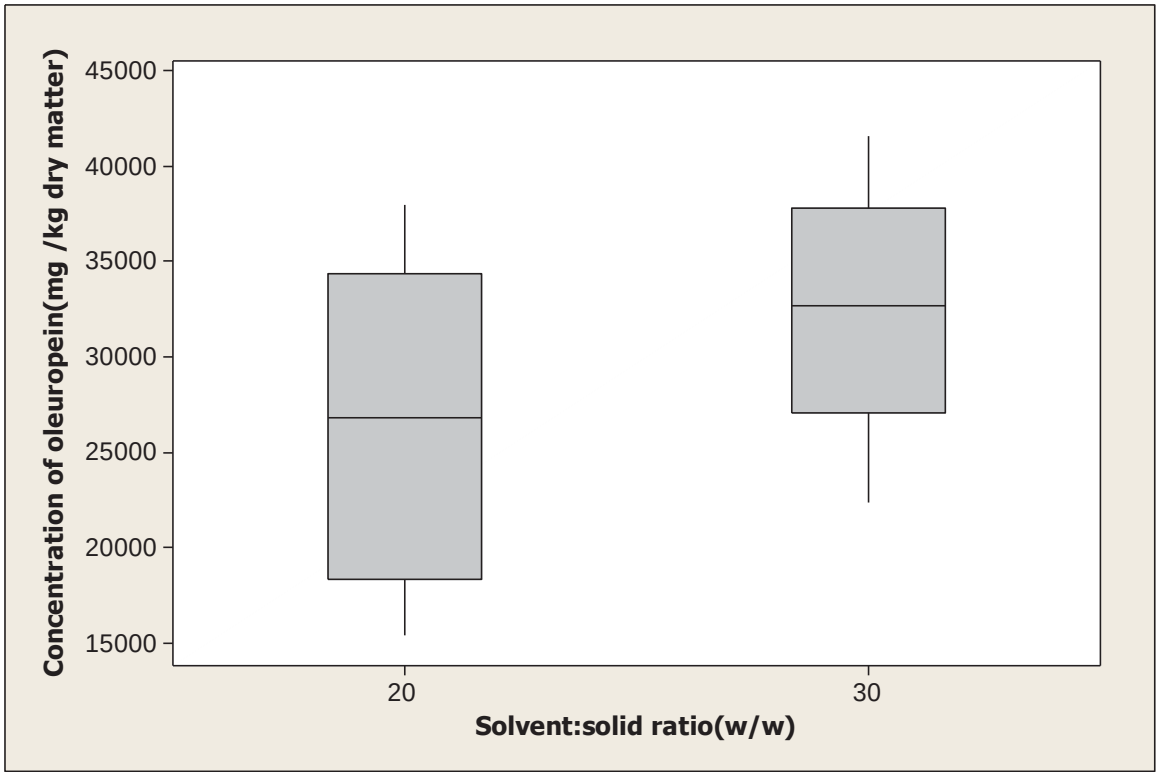



Figure 4.5.b: Boxplot of Oleuropein Concentration vs. Solvent: Solid Ratio, All Experiments Were Carried out in Triplicates and Data Points Are Mean Value of Triplicates (N=3)

### 4.2.5 The Effect of Ethanol Concentration on Extraction Yield

Changes in ethanol concentration can modify the physical properties of the solvent and solubility of phenolics compounds (Cacace & Mazza 2003). The influence of ethanol concentration on extraction yield was investigated. Ethanol concentrations of 60%, 70% and 80% (v/v) were used for this work, which were the concentrations mostly reported in previous studies (Bouaziz & Sayadi, 2005; Japon-Lujan et al., 2006; Jemai, 2008; Kiritsakis et al., 2010).

Analysis of variance (ANOVA) for total phenolics (see Appendix 4.1) indicates that total phenolics recovered from olive leaves was not significantly affected by ethanol concentration ($P \geqslant 0.05$). However, ethanol concentration had a significant impact ($P \leqslant 0.05$) on the oleuropein concentration in olive leaf extracts (see Appendix 4.2). Figures 4.6.a and 4.6.b were created by plotting the mean values of three measurements for 36 extractions against ethanol concentrations. As shown in Figure 4.6.a, there was no significant difference ($P \geq 0.05$) in total phenolics between extracts obtained using ethanol at three different concentrations. In terms of oleuropein, there was significant difference ($P \leq 0.05$) between the extracts. Increasing amount of oleuropein was recovered with increasing ethanol concentration as shown in Figure 4.6.b. The highest concentration of

oleuropein was obtained in 80% ethanol extract, which is 19% higher than that of 60% ethanol extract.

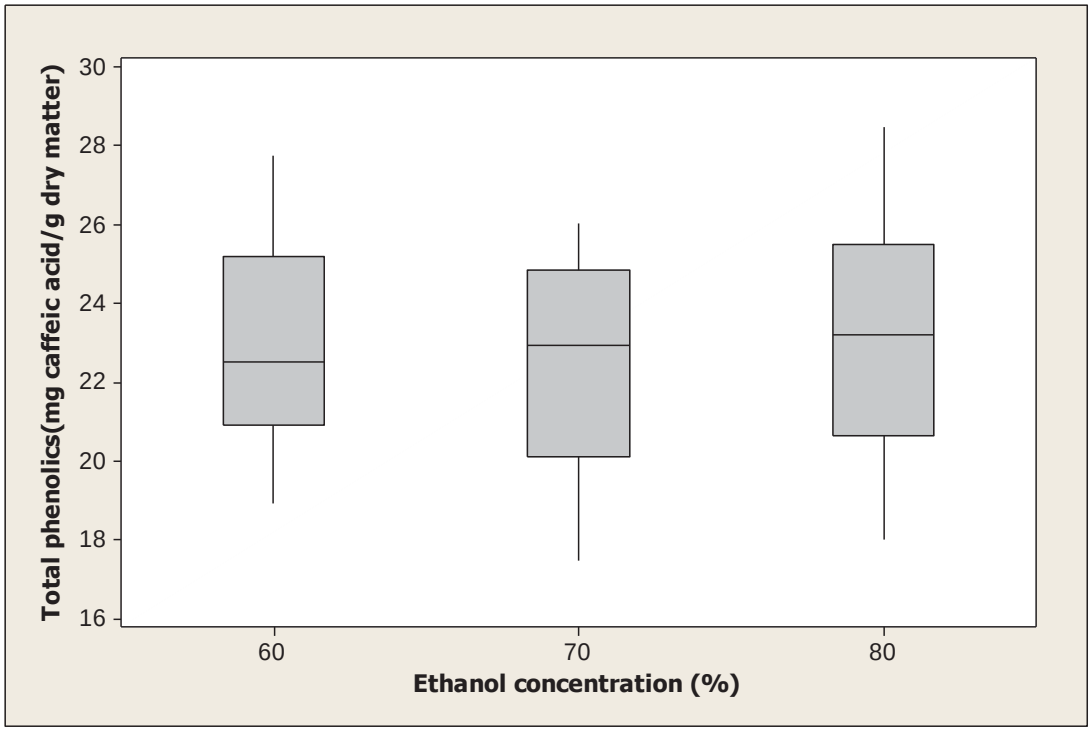



Figure 4. 6.a: Boxplot of Total Phenolics vs. Ethanol Concentration, All Experiments Were Carried out in Triplicates and Data Points Are Mean Value of Triplicates (N=3)

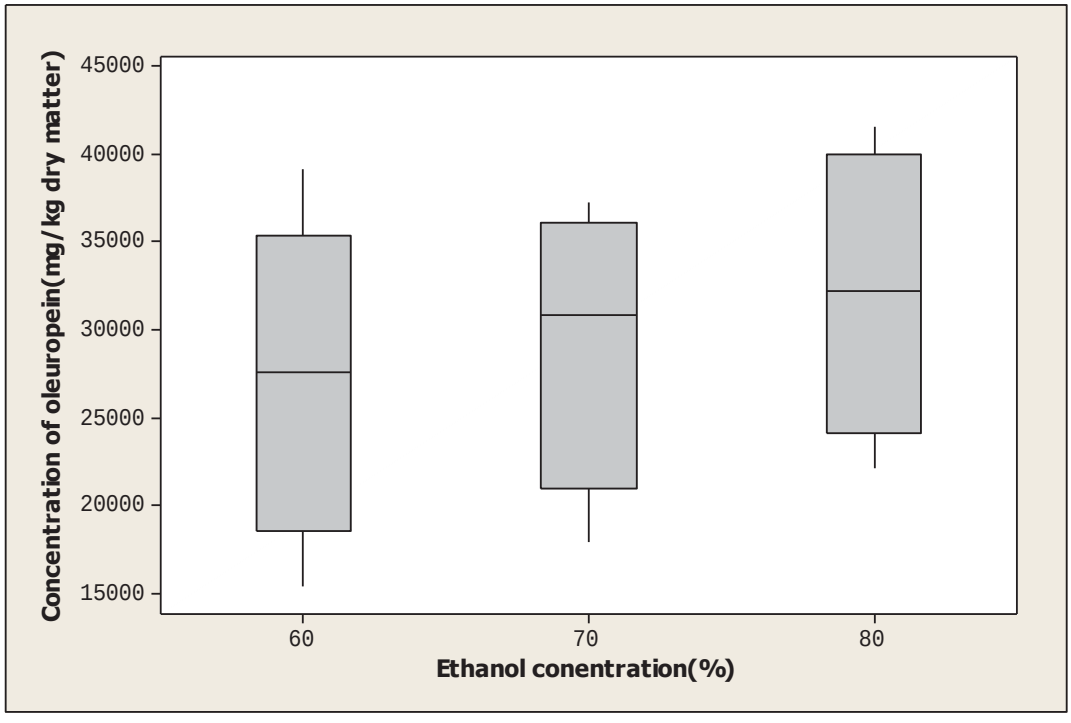



Figure 4.6.b: Boxplot of Oleuropein Levels vs. Ethanol Concentration, All Experiments Were Carried out in Triplicates and Data Points Are Mean Values ±SEM of Triplicates (N=3)

The optimum ethanol concentration for olive leaf extraction reported in previously published literatures varied. Japon & De Castro (2006b) suggested 80% aqueous ethanol (v/v) was the optimum solvent for extraction of the targeted phenolics from olive leaf (Japon-Lujan & De Castro, 2006b). Mylonaki et al. (2008) used 40%, 50% and 60% ethanol (v/v) to extract phenolic compounds from olive leaves, and they discovered that the ethanol concentration had a biphasic effect, as an intermediate ethanol level (50%) appeared to provide the lowest yield. Higher concentration of total phenolics was obtained in extracts using 40% and 60% ethanol (v/v). It was suggested that decreasing or increasing the ethanol concentration both favoured extraction of phenolics from olive leaves. The maximum amounts were obtained at an ethanol concentration of 60%. Mylonaki et al. (2008) gave possible reasons for such a biphasic tendency in response to ethanol changes. Many of the olive leaf phenolics are glycosides and they are more polar and water soluble, hence a higher extract can be obtained with low concentration of ethanol. On the other hand, oleuropein, which is significantly less polar, would be more soluble in higher concentrations of ethanol (Mylonaki et al., 2008). It may explain though the highest concentration of oleuropein was obtained in 80% ethanol extract in this study, but there may be more polar and water soluble phenolic compounds present in 60% and 70% ethanol extracts, hence no significant difference was found between 60%, 70% and 80% ethanol extracts in terms of total phenolic content.

## 4.3 Extraction of Olive Leaf Phenolics Using Boiling Water

Boiling water was used to extract phenolic compounds from olive leaves by few researchers (Silva et al., 2006; Pereira et al., 2007; Malik & Bradford, 2008). It was suggested boiling of dried leaves was a very efficient method for extracting oleuropein (Malik & Bradford, 2008). In this study, olive leaf extracts were recovered by boiling 5 gram dried olive leaf powder in 100 ml or 150 ml water. Table 4.1 shows total phenolics and oleuropein concentrations obtained in the two boiling water extracts. The higher the solvent: solid ratio produced the significantly higher ($P \leq 0.05$) the total phenolics and oleuropein concentrations. This confirmed that the solvent: solid ratio has a positive effect on the extraction yield. It is notable that 50% less phenolic compounds were obtained when water was used as a solvent compared to ethanol as a solvent. There was nearly 67% less oleuropein in water extracts than ethanol extracts. Despite infusion being a traditional method of making herbal medicine (Chevallier, 2000), and it has advantage of being simple

and low cost; however it produced extracts with much lower concentration of important phenolic compounds and there may be degradation of those compounds during boiling.

Table 4. 1: The Total Phenolics Content and Oleuropein Levels of Olive Leaf Extract Using Boiling Water

| **Extraction conditions** | **Solvent: solid ratio (20:1)** | **Solvent: solid ratio (30:1)** |
|---|---|---|
| Total phenolics content *(mg caffeic acid/g dry matter) | 13.39±0.13 | 16.51±0.84 |
| Oleuropein concentration*(mg/ kg dry matter) | 13225±546 | 18694±241 |

*Results are expressed as mean value ± SEM (n=3)

## 4.4 Ultrasonic Assisted Solvent Extraction of Olive Leaf Phenolics

As a novel extraction technique, the use of ultrasound sonication can offer advantage of reduced extraction time and improved efficiency (Jerman et al., 2010). All these benefits are attributed to acoustic cavitation, which can disrupt plant cells and lead to increased diffusion of cell contents into the extraction solution, consequently enhancing mass transfer (Jerman et al., 2010). The two most common ultrasonic techniques that are used for extraction are the ultrasonic bath and the probe system (Chemat et al., 2009). Despite ultrasound baths being widely used, an ultrasonic probe can offer more efficient cavitation in the solution (Chemat et al., 2009).

Ultrasonic assisted solvent extraction in this work was carried out in an ultrasonic bath using the same operational parameters optimised earlier in Section 4.2.2 (40±1C°, solvent: solid ratio: 30:1), a total of 45 minutes and 60% or 80% ethanol (v/v) were used to investigate the possible effect of ethanol concentration on the extraction yield. As can be seen in Table 4.2, There was no significant difference ($P \leq 0.05$) in total phenolic content between 60% ethanol extracts (v/v) and 80% ethanol extracts (v/v); however, significantly higher amounts of oleuropein ($P \leq 0.05$) were obtained in the 80% ethanol extract (v/v). Nearly 30% higher concentration of oleuropein was recovered using 80% ethanol than 60% ethanol. This confirmed that ethanol concentration had an impact on oleuropein yield. Increasing ethanol may enhance extraction of oleuropein, while it may not significantly affect total phenolic content. Le et al. (1998) reported that total phenolics recovered were 16.8mg caffeic acid equivalent per gram olive leaves using absolute methanol under sonication for 75 minutes (Le et al., 1998).

Table 4. 2: The Total Phenolics Content and Oleuropein Concentrations of Olive Leaf Extract Using Ultrasonication

| Extraction condition | Ethanol concentration (60%) | Ethanol concentration (80%) |
|---|---|---|
| Total phenolics content *(mg caffeic acid/g dry matter) | 26.46±0.92 | 25.91±0.78 |
| Oleuropein concentration*(mg/ kg dry matter) | 24488±958 | 37511±382 |

*Results are expressed as mean value ± SEM (n=3)

The results demonstrate that ultrasound can dramatically accelerate extraction rate and reduce the extraction time. By using an ultrasound bath, a similar amount of phenolic compounds was recovered in 45 minutes; in contrast, it needed 24 hours to reach completion with conventional extraction (Section 4.2.2). This is in agreement with findings by Japon et al., (2006), who proposed a dynamic ultrasound-assisted extraction for olive leaf bioactive phenolics compounds, complete extraction of the target analytes was achieved in 25 minutes (Japon-Lujan et al., 2006). Jeman et al. (2010) also developed a new method for extraction of olive fruits phenols using high intensity probe ultrasonication and suggested that the method was more efficient in comparison to conventional solvent extraction, with up to 80% enhancement in the case of oleuropein yield (Jeman et al., 2010).

## 4.5 Comparison of Three Proposed Extraction Methods

The three proposed methods were compared in terms of extraction efficiency compared to the method using 80% methanol under optimised extraction condition (40±1°C for 24 hours, solvent: solid ratio, 30:1) (Table 4.3). Several publications reported methanol was the best solvent for extraction of phenolics from olive leaf (Le et al., 1998; Bouaziz & Sayadi, 2005; Malik & Bradford, 2008). Le et al. (1998) compared hexane, diethyl ether, ethyl acetate and methanol and revealed that the higher the solvent polarity, the higher the phenol extraction yields. Methanol gave the best performance amongst all four solvents in terms of phenol extraction yield (Le et al., 1998)

Bouaziz & Sayadi (2005) compared different yield of recovered oleuropein by treating same quantity of olive leaf samples with 50%, 80% methanol (v/v), 50% ethanol (v/v), absolute ethanol and methanol, they reported the highest amount of oleuropein was obtained using 80% methanol (v/v) as the extraction mixture. Malik & Bradford (2008) also reported that extraction with 80% methanol (v/v) was the most effective method for

olive leaf polyphenols for laboratory used (Malik & Bradford, 2008). However, methanol is not considered as a solvent for making extracts for human consumption due to possible existence of toxic residue in extracts. Hence ethanol and water was used as solvent for extraction of phenolic compounds in this study and the extraction efficiency was evaluated.

Table 4. 3: Comparison of Extraction Efficiency of Three Proposed Extraction Method for Olive Leaf Phenolics

| **Extraction method** | **Control method** | **Proposed method one (conventional method)** | **Proposed method two (boiling water)** | **Proposed method three (ultrasonic bath)** |
|---|---|---|---|---|
| Solvent & concentration (v/v) | 80% aqueous methanol | 80% aqueous ethanol | Boiling water | 80% aqueous ethanol |
| Extraction temperature | 40±1°C | 40±1°C | 100°C | 40±1°C |
| Extraction time | 24hours under agitation | 24hours under agitation | 10 minutes | 45 minutes in ultrasonic bath |
| Solvent: solid ratio | 30:1 | 30:1 | 30:1 | 30:1 |
| Total phenolics content *(mg caffeic acid/g dry matter) | 26.10±0.14 | 25.25±0.46 | 16.51±0.84 | 25.91±0.78 |
| Oleuropein concentration*(mg/ kg dry matter) | 45913±660 | 41987±309 | 18694±241 | 37511±382 |
| Extraction efficiency (%) on basis of total phenolics | 100 | 97.54 | 63.25 | 99.27 |
| Extraction efficiency (%) on basis of oleuropein | 100 | 91.45 | 40.71 | 81.70 |

*Results are expressed as mean value ± SEM (n=3)

As can be seen in the Table 4.3, the highest amount of total phenolics and oleuropein was achieved using 80% methanol (v/v). Ultrasound bath did not significantly enhance the total phenolic content and oleuropein ($P \geq 0.05$) recovered compared with ethanol extraction. The least total phenolic and oleuropein recovered was by boiling water.

The extraction efficiency of three proposed extraction methods was determined by comparing extraction yield (total phenolics & oleuropein) of each method with "maximum extraction yield", which was determined by control method (80% methanol, 40°C, 24hours, solvent: solid ratio: 30:1). Extraction efficiency was calculated as a percentage of yields of each extraction method with respect to maximum yield.

Both ultrasonic bath and ethanol extraction can achieve over 95% recovery of total phenolics, whereas boiling water only achieves 63.25% recovery. In the case of oleuropein, recoveries with ethanol were 50% higher than boiling water extraction and 10% higher than ultrasonic bath extraction. These findings indicate that the ethanol water mixture was more effective solvent than water alone for extraction of phenolics from olive leaves. Ultrasound can provide a better mixing between leaf cells and extraction solution than conventional agitation. The advantage of ultrasonic bath extraction and boiling water infusion was very simple to perform in a much shorter time. To summarise, comparing different extraction methods, the ethanol extraction method resulted in highest recovery and it could offer a sufficient recovery of phenolic compounds from olive leaf. Ultrasound assisted extraction could be a very promising technique for recovery of olive leaf phenolics, however, some important parameters associate with ultrasound needs to be further optimized such as ultrasound radiation amplitude.

The aim of the study was to determine a suitable method which can be used for extraction of phenolic compounds from olive leaves. Method and solvent selection was the first concern for this project and then followed by determination of optimum extraction parameters for the method. The ideal extraction procedure should be fast, simple and reproducible if it is to be performed repeatedly, and hence solvent extraction reported previously was selected as a method in this study.

Extraction can be “selective” or “total”. The initial choice of the most appropriate solvent for extraction is based on its selectivity for the substance to be extracted. In a selective extraction, the plant materials are extracted using a solvent of appropriate polarity following the principle of “like dissolve like”. If an extraction is referred to as “total”; a polar organic solvent is employed in an attempt to extract as many compounds as possible. This is based on the ability of alcoholic solvents to increase cell wall permeability; facilitating the efficient extraction of large amount of polar, medium to low polarity constitutes. The solvent should be low toxicity, economical and easily recycled by evaporation (Seidel, 2006). In this work, extraction can be regarded as total extraction; ethanol and water mixture was selected as the solvent.

Solvent extraction is a process designed to separate soluble compounds by diffusion from a solid matrix using a liquid matrix. This process takes place by two steps, which are the

adsorption of solvent into the solid phase by osmotic forces, by capillarity and by solvation of the ions in the cells, then followed by diffusion from the solid phase (Escribano-Bailon & Santos-Buelga 2003). The resulting extraction is a function of how fast the compound is dissolved and how easily the equilibrium in the liquid is reached (Pinelo et al., 2005).

It was found in this study that temperature, the solvent: solid ratio, ethanol concentrations are the factors which greatly affect extraction of phenolic compounds from olive leaves ($P \leq 0.05$), increasing temperature and the solvent: solid ratio enhanced extraction of total phenolic compounds from dried olive leaves; increasing ethanol concentration favoured the extraction of oleuropein.

## 4.6 HPLC Phenolic Profile of Olive Leaf Extract

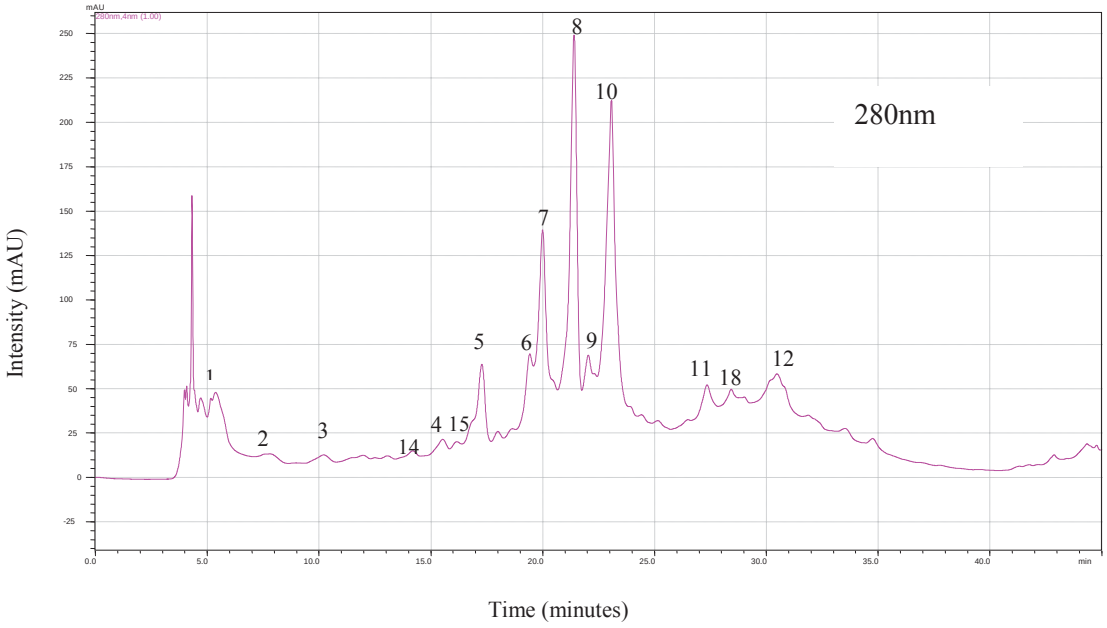
### 4.6.1 HPLC Phenolic Profile of 80% Aqueous Ethanol Olive Leaf Extract

In order to obtain an insight into the composition of the olive leaf extracts, HPLC-DAD analyses were undertaken. The detection was recorded at 245nm, 280nm, 330nm, 340nm and 350nm. The HPLC profiles of phenolic compounds present in the 80% aqueous ethanol olive leaf extract are shown in Figure 4.7. The retention time and percentage of the main compounds in the total extract are shown in Table 4.4. A total of 15 compounds were identified, the most abundant compounds in the ethanol based olive leaf extract are oleuropein, followed by luteolin-4-O- glucoside (peak 9), luteolin-7-O-glucoside (peak 7), apignin-7-O-glucoside (Peak 10) and rutin (peak 6). Oleuropein (peak 8) accounts for 44.31% of total leaf phenolic contents, other four compounds account for 12.63%, 11.72%, 8.85% and 8.24% of total leaf phenolic contents respectively. The phenolic compounds present in trace amount were vanillin, o-coumaric acid and p-coumaric acid. Three peaks (16, 17, 18) detected in the extracts were not identified.

Table 4. 4: Retention Time and Abundance of the Main Phenolic Compounds Present In 80% Aqueous Ethanol Olive Leaf Extracts.

| Peak no | Compound Name | Retention time (minutes) | Concentration (mg/kg dry matter) | % Absolute |
|---|---|---|---|---|
| 1 | Hydroxytyrosol | 5.5 | 919±60 | 1.79 |
| 2 | Tyrosol | 7.3 | 312±8 | 0.61 |
| 3 | Caffeic acid | 10.3 | 75±7 | 0.15 |
| 4 | Ferulic acid | 15.7 | 524±19 | 1.02 |
| 5 | Verbascoside | 17.4 | 2406±117 | 4.70 |
| 6 | Rutin | 19.4 | 4221±75 | 8.24 |
| 7 | Luteolin-7-O-glucoside | 20.1 | 6003±63 | 11.72 |
| 8 | Oleuropein | 21.4 | 22708±168 | 44.31 |
| 9 | Luteolin-4-O-glucoside | 23.1 | 6471±209 | 12.63 |
| 10 | Apigenin-7-O-glucoside | 23.3 | 4537±99 | 8.85 |
| 11 | Luteolin | 27.3 | 820±141 | 1.60 |
| 12 | Apigenin | 30.1 | 2245±141 | 4.38 |
| 13 | Vanllin | 12.0 | | ≤0.02 |
| 14 | P-coumaric acid | 13.6 | | ≤0.02 |
| 15 | O-coumaric acid | 17.2 | | ≤0.02 |
| 16 | unknown | 13 | | |
| 17 | unknown | 14 | | |
| 18 | unknown | 28.5 | | |

* Concentrations are expressed as mean ±SEM (n=3), absolute% was expressed as percentage of individual phenolic compounds in the total extracts



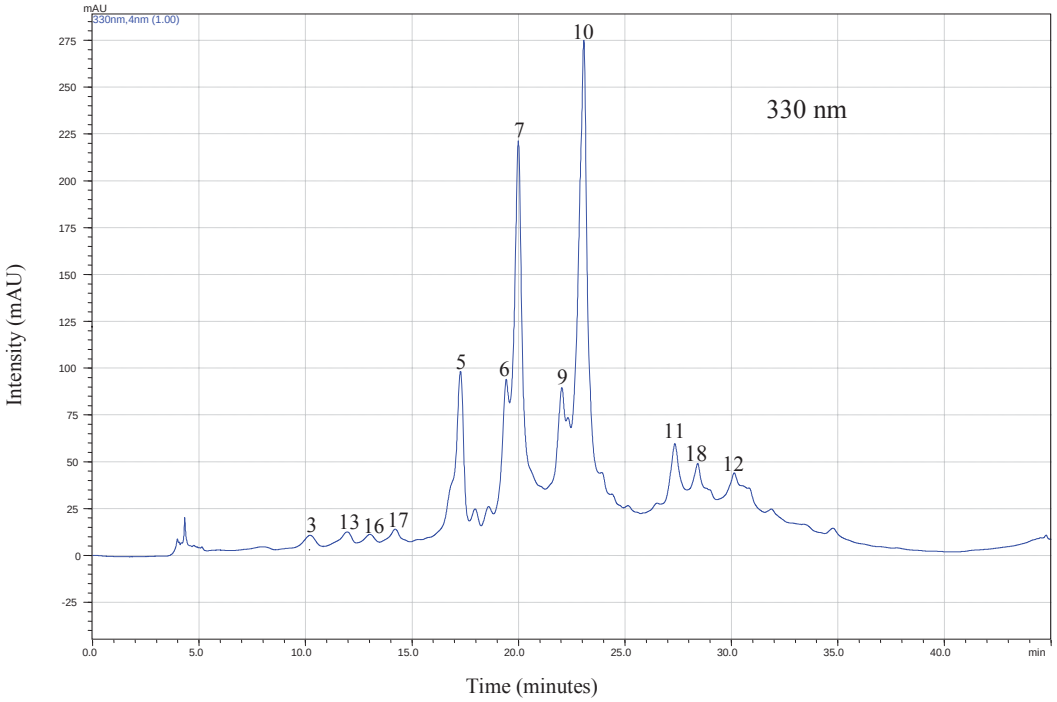




Figure 4. 7: HPLC Profile of Olive Leaf Extract ('Frantoio' Cultivar) Obtained By 80% Aqueous Ethanol At Solvent:Solid Ratio of 30 and Temperature of 40±0.5°C (1) hydroxytyrosol; (2 ) tyrosol; (3) caffeic acid; (4) ferulic acid; (5) verbascoside; (6) rutin; (7) luteolin 7-O-glucoside; (8) oleuropein; (9) luteolin-4-O-glucoside; (10) apigenin 7-O-glucoside; (11) luteolin; (12) apigenin; (13) vanillin; (14) p-coumaric acid; (15) o-coumaric acid; (16) (17) (18) unknown . Detection was at (a) 280nm and (b) 330nm

### 4.6.2 HPLC Phenolic Profile of 80% Aqueous Methanol Olive Leaf Extract

The chromatogram in Figure 4.8 illustrates the phenolics composition of olive leaf extract obtained using 80% aqueous methanol as solvent under optimum extraction conditions (solvent: solid ratio: 30; temperature: 40±0.5°C). The percentage of each phenolic compound in this extract is given in Table 4.5. The same group of phenolic compounds as those found in the ethanol based extract were observed in methanol based extract. Oleuropein (peak 8), along with luteolin-7-o-glucoside (peak 7), luteolin-4-o-glucoside (peak 9), apigenin-7-o-glucoside (peak 10) and rutin (peak 6) were identified as major components in extract with percentage of 38.56%, 19.58%, 11.95%, 8.48% and 7.57% of total leaf phenolic contents respectively. Vanillin, o-coumaric acid and p-coumaric acid, vallin acid were detected in trace amounts.

Table 4. 5: Retention Time and Abundance of the Main Phenolic Compounds Present In 80% Aqueous Methanol Olive Leaf Extract

| Peak no | Compound Name | Retention time (minutes) | Concentration (mg/kg dry matter) | % Absolute |
|---|---|---|---|---|
| 1 | Hydroxytyrosol | 5.5 | 1908±145 | 3.21 |
| 2 | Tyrosol | 7.3 | 567±16 | 0.95 |
| 3 | Caffeic acid | 10.3 | 80.95±0.27 | 0.14 |
| 4 | Ferulic acid | 15.7 | 62.99±0.27 | 0.11 |
| 5 | Verbascoside | 17.4 | 2698±50 | 4.54 |
| 6 | Rutin | 19.3 | 4505±190 | 7.57 |
| 7 | Luteolin-7-O-glucoside | 20.1 | 11647±115 | 19.58 |
| 8 | Oleuropein | 21.4 | 22933±534 | 38.56 |
| 9 | Luteolin-4-O-glucoside | 23.1 | 7105±51 | 11.95 |
| 10 | Apigenin-7-O-glucoside | 23.3 | 5045±50 | 8.48 |
| 11 | Luteolin | 27.3 | 854±8 | 1.44 |
| 12 | Apigenin | 30.1 | 2070±23 | 3.48 |
| 13 | Vanllic acid | 9.8 | | ≤0.02 |
| 14 | Vanllin | 12.0 | | ≤0.02 |
| 15 | P-coumaric acid | 13.6 | | ≤0.05 |
| 16 | O-coumaric acid | 17.2 | | ≤0.05 |
| 17 | unknown | 13 | | |
| 18 | unknown | 14 | | |
| 19 | unknown | 28.5 | | |

* Concentrations are expressed as mean ±SEM (n=3), absolute% was expressed as percentage of individual phenolic compounds in the total extract

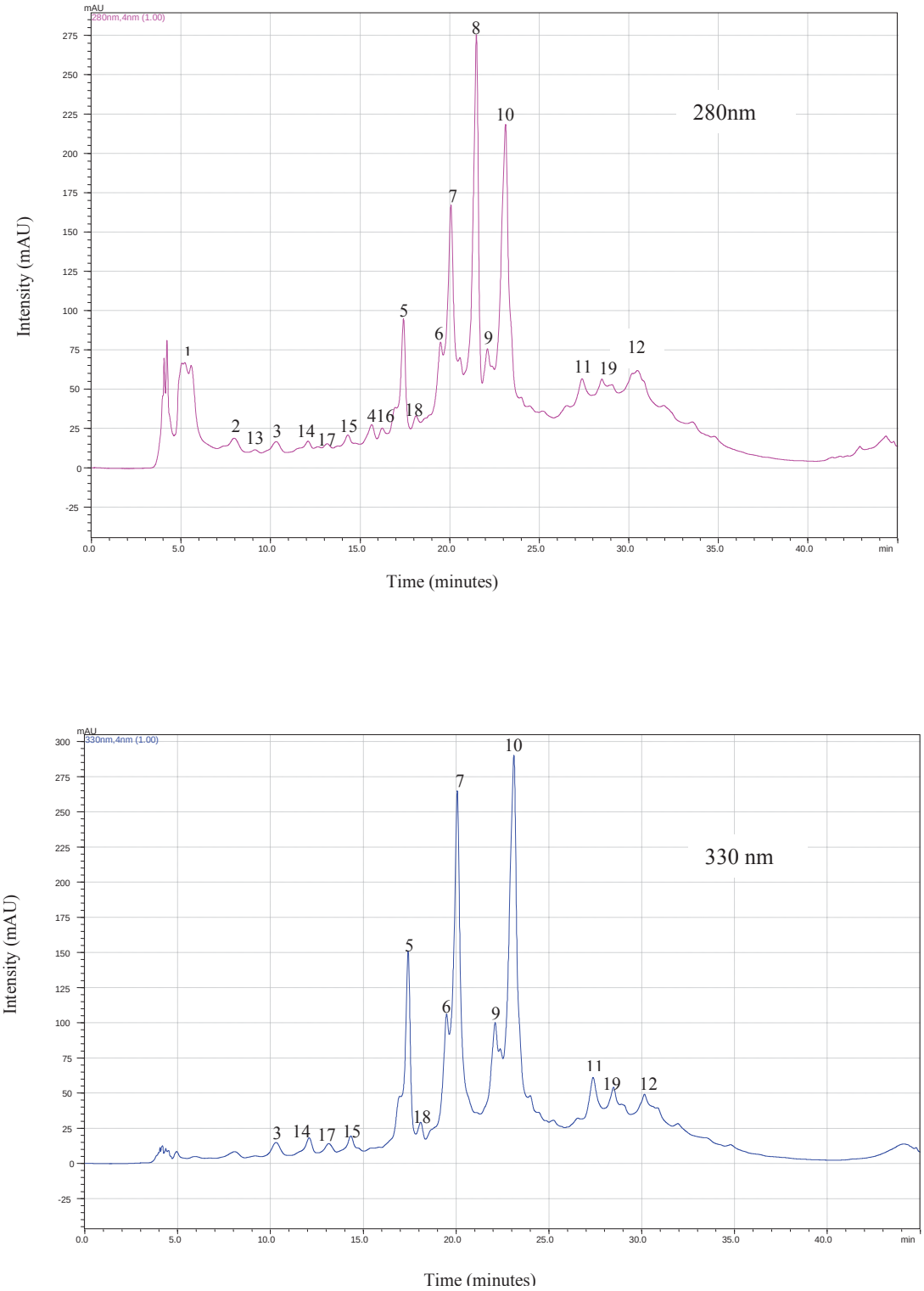



Figure 4. 8: HPLC Profile of Olive Leaf Extract ('Frantoio' Cultivar) Obtained By 80% Aqueous Methanol At A Solvent: Solid Ratio of 30 and Temperature of 40±0.5°C. (1) hydroxytyrosol; (2) tyrosol; (3) caffeic acid; (4) ferulic acid; (5) verbascoside; (6) rutin; (7) luteolin 7-O-glucoside; (8) oleuropein; (9) luteolin-4-O-glucoside; (10) apigenin 7-O-glucoside; (11) luteolin; (12) apigenin; (13) vanllic acid; (14) vanillin; (15) p-coumaric acid; (16) o-coumaric acid; (17)(18)(19) unknown. Detection was at (a) 280nm and (b) 330nm

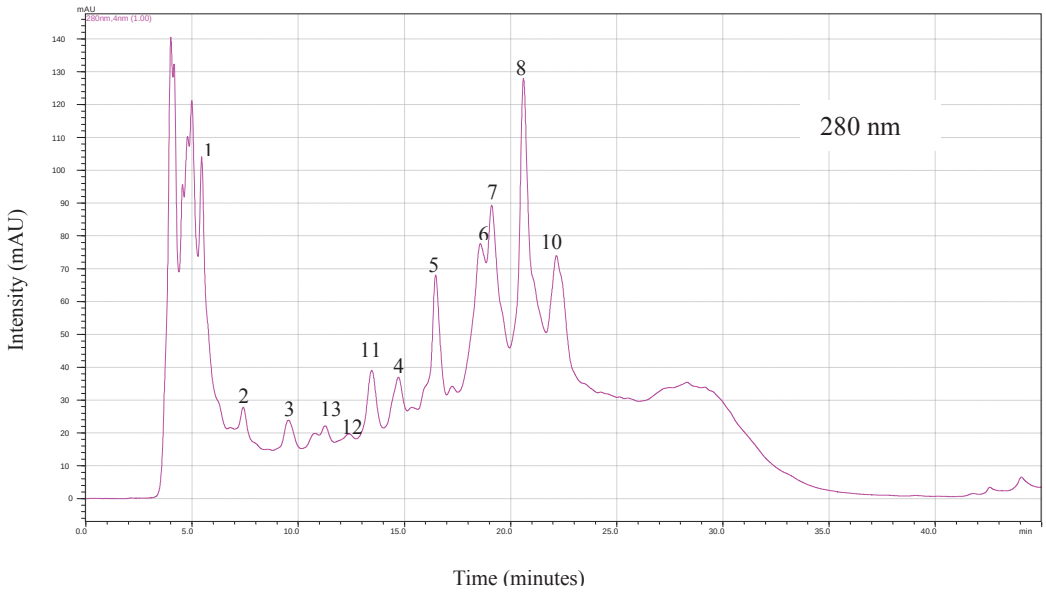
### 4.6.3 HPLC Phenolic Profile of Olive Leaf Extracts Using Boiling Water

The HPLC-DAD analysis of olive leaf extract using boiling water only allowed the identification of 12 phenolic compounds (Figure 4.9): hydroxytyrosol, tyrosol, caffeic acid, p-courmaric acid, ferulic acid, verbascoside, oleuropein, luteolin 7-O-glucoside, rutin, apigenin-7-O-glucoside and luteolin 4-O-glucoside. The olive leaf extract using boiling water exhibited a profile in which oleuropein (Peak 8) was the compound present in highest amount, representing 35.71% of total identified compounds (Table 4.6), and caffeic acid was the minor compound, corresponding to 0.53% of total phenolics. Other four major phenolics compounds in olive leaf infusion are luteolin 7-O-glucoside (26.21%), luteolin 4-O-glucoside (10.84%), verbascoside (6.67%) and rutin (7.33%). Vanillin was detected in trace amount (≤0.02).

Table 4. 6: Retention Time and Abundance of the Main Phenolic Compounds Present In Olive Leaf Infusion

| Peak no | Compound Name | Retention time (minutes) | Concentration (mg/kg dry matter) | % Absolute |
|---|---|---|---|---|
| 1 | hydroxytyrosol | 5.5 | 411±11 | 1.75 |
| 2 | Tyrosol | 7.3 | 222±27 | 0.95 |
| 3 | Caffeic acid | 10.3 | 124±22 | 0.53 |
| 4 | Ferulic acid | 15.7 | 395±6 | 1.68 |
| 5 | Verbascoside | 17.4 | 1564±16 | 6.67 |
| 6 | Rutin | 19.4 | 1720±226 | 7.33 |
| 7 | Luteolin-7-O-glucoside | 20.1 | 6146±48 | 26.21 |
| 8 | Oleuropein | 21.4 | 8375±105 | 35.71 |
| 9 | Luteolin-4-O-glucoside | 23.1 | 2543±16 | 10.84 |
| 10 | Apigenin-7-O-glucoside | 23.4 | 1136±187 | 4.84 |
| 11 | P-Coumaric acid | 13.6 | 509±6 | 2.17 |
| 12 | Vanllin | 12.0 | | ≤0.02 |
| 13 | unknown | 6.1 | | |
| 14 | unknown | 11.2 | | |

*Concentrations are expressed as mean ±SEM (n=3), absolute% was expressed as percentage of individual phenolic compounds in the total extract



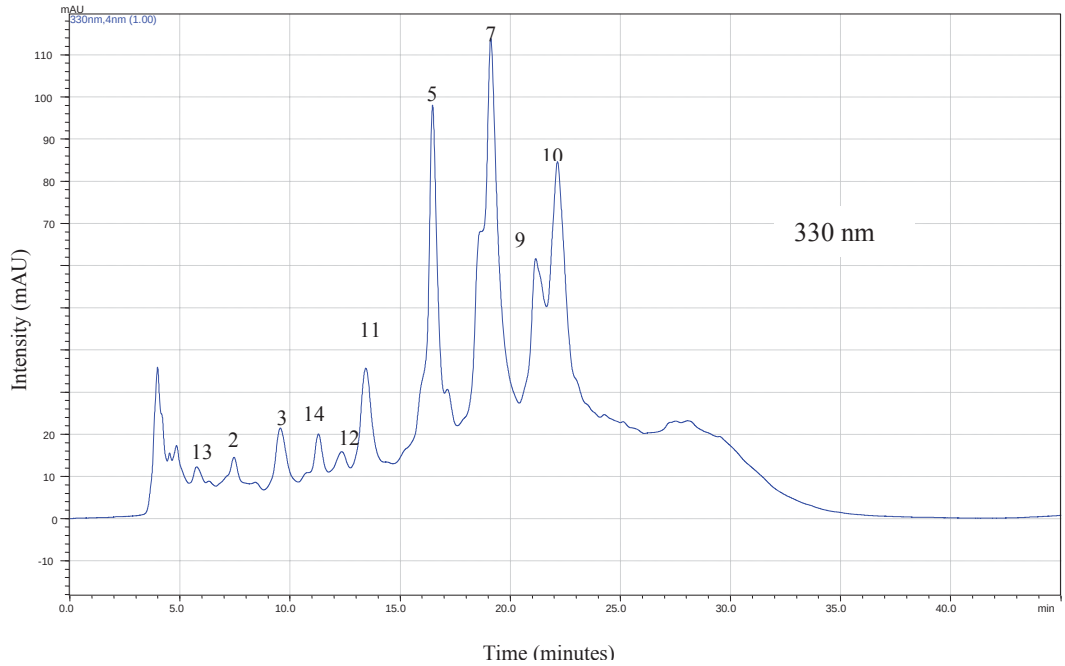




Figure 4. 9: HPLC Profile of Olive Leaf Extract ('Frantoio' Cultivar) Obtained By Boiling Water (1) Hydroxytyrosol; (2) tyrosol; (3) caffeic acid; (4) ferulic acid; (5) verbascoside; (6) rutin; (7) luteolin 7-O-glucoside; (8) oleuropein; (9) luteolin-4-O-glucoside; (10) apigenin 7-O-glucoside; (11) p-Coumaric acid Detection was at 280nm and 330nm

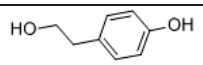
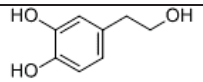
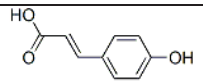
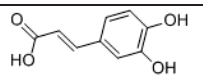
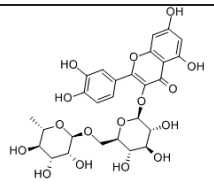
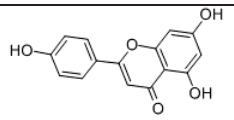
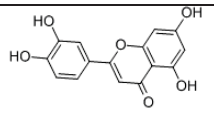
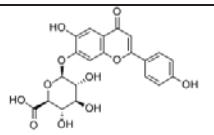
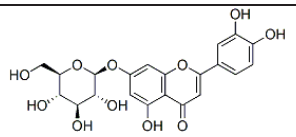
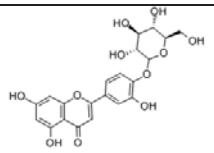
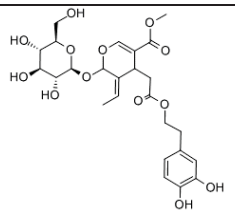
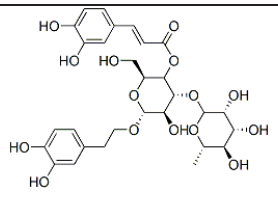
### 4.6.4 Phenolic Compounds in Olive Leaf Extract

The results above (Tables 4.4-4.6) revealed that there were six main phenolic compounds in the olive leaf extracts. They were oleuropein, hydroxytyrosol, luteolin-7-glucoside, luteolin-4-glucoside, apigenin-7-O-glucoside, verbascoside. These compounds were also found by others in the olive leaf extracts (Benavent et al., 2000; Briante et al., 2002; Bouzaiz & Sayadi, 2005; Japon-Lujan et al., 2006; Pereria et al., 2007; Altiok et al., 2008;). Benavent et al. (2000) reported that oleuropein was the largest fraction present (24.5%) while other phenolics compounds such as hydroxytyrosol (1.5%), luteolin-7-glucoside (1.4%), verbascoside (1.1%), tyrosol (0.7%), and apigenin-7-O-glucoside (1.4%) were also isolated from the leaves. Atiok et al. (2008) reported the abundance of oleuropein (29%) in an olive leaf crude extract (Atiok et al., 2008). Bouzaiz & Sayadi (2005) reported that six flavonoid (luteolin 7-*O*-glucoside, luteolin 7-*O*-rutinoside, apigenin 7-*O*-glucoside, rutin, luteolin and apigenin) present in Chemlai olive leaf extract (Bouzaiz & Sayadi, 2005). Pereria et al. (2007) quantified oleuropein and lueolin-7-O-glucoside as the most abundant phenolic compounds present in a lyophilised olive leaf extract (Pereria et al., 2007). Meirinhos et al. (2005) quantified eight flavonoid compounds in olive leaf samples and reported luteolin-4-O-glucoside was the major flavonoid compound for Portuguese olive leaf cultivars (Meirinhos et al., 2005).

There were four groups of phenolic compounds identified in the leaf extracts in this study, namely phenolic alcohols (e.g. hydroxytyrosol, tyrsol), phenolic acid (e.g. caffeic acid, P-coumaric acid), flavonoids (e.g. luteolin-7-O-glucoside, luteolin-4-O-glucoside, and apigenin-7-O-glucoside) and secoiridoids (e.g. oleuropein, verbascoside). These compounds are either secondary metabolites or compounds formed during leaf storage and extraction process (Salvador & Fregapane, 2010). The structures of all phenolic compounds identified in the three olive leaf extracts are shown in Table 4.7.

Oleuropein, the most abundant secoiridoids present in *O. europaea* fruits and leaves, is a hydroxytyrosol ester with a β-glycosylated elenolic acid (Briante et al., 2001). Secoiridoids are present exclusively in plants that belongs to the *Oleaceae* family (Silva et al., 2009).The formation of oleuropein is related to the iridoids which are produced via secondary metabolism of monoterpenes as precursors of various indole alkaloids. The leaf has been regarded as the primary site of plant metabolism at the level of both primary and secondary plant products (Ryan et al., 2002). Verbascoside is another secoiridoid derived from hydrotyrosol backbone (Laguerre et al., 2009).

Table 4. 7: Phenolic Compounds Present In Crude Olive Leaf Extracts and Their Chemical Structures, Reproduced From (Macdonald-Wicks et al., 2006)

| **Class** | **Compound** | **Chemical Structure** |
|---|---|---|
| Phenyl alcohol | Tyrosol |  |
| | Hydroxytyrosol |  |
| Phenyl acid | P-coumaric acid |  |
| | Caffeic acid |  |
| Flavonoids | Rutin |  |
| | Apigenin |  |
| | Luteolin |  |
| | Apigenin-7-O-gluocoside |  |
| | Luteolin-7-O-glucoside |  |
| | Luteolin-4-O-glucoside |  |
| Secoiridoids | Oleuropein |  |
| | Verbascoside |  |

Hydroxytyrosol and tyrosol are the most abundant phenolic alcohol in olives. Hydroxytyrosol is rarely in the free form in nature with the exception of ripened olives where it occurs through the hydrolysis of oleuropein (De Leonardis et al., 2008).

The dominant flavonoids present in the olive leaf extracts were flavonoid glycosides such as luteolin-7-O-glucoside, luteolin-4-O-glucoside, apigenin-7-O-glucoside and rutin. Other flavonoids present in olive leaves are in aglycone forms which include apigenin and luteolin (Laguerre et al., 2009).

There are three enzymes involved in metabolism of major phenolic compounds in olive fruits and leaves. The enzyme involved in accumulation and degradation of oleuropein is β-glucosidase, which plays an important role in the anabolic and catabolic routes of oleuropein biosynthesis (Gutierrez-Rosales, 1998). Phenylalanine ammonialyase (PAL) is a regulatory enzyme involved in the biosynthesis of a large group of phenylpropanoid-derived secondary products such as flavonoids, isoflavonoids, coumarins, lignins and other phenolic compounds such as tyrosol (Gutierrez-Rosales, 1998). Polyphenol oxidase (PPO) is an enzyme involved in olive fruit and leaf browning. The browning occurs when phenols are oxidized to highly reactive quinones, which then polymerize. This oxidation reaction is catalysed by PPO in presence of oxygen. The substrates for the reaction are simple phenols derived from oleuropein. Polyphenol oxidase activity was significantly higher in fruits than in leaves and it increased during fruit ripening (Ortega-Garcia., 2008).

A wide range of phenolic compounds were identified in the olive leaf extracts. Variability in the type and amount of phenolic compounds in olive leaf extract may be due to geographical, varietal, seasonal factors and different extraction methods applied (Hayes et al., 2010).The phenolic compounds in the olive leaf extracts may be formed during sample preparation and extraction. The chemical and enzymatic reactions may take place during this process including hydrolysis of glycosides by β-glucosidase, oxidation of phenolic compounds polyphenol oxidase and polymerization of free phenols.

Laguerre et al. (2009) discovered that mature olive leaf extract contained higher levels of verbascoside isomers and glycosylated forms of luteolin, while young ones presented higher contents of oleuropein , ligstroside and flavonoid aglycones. They suggested that there were two main bioconversion scenarios which may occur during maturation of olive leaves (1) a bioconversion of oleuropein and ligstroside into verbascoside isomers and oleuroside, and (2) a bioconversion of flavonoid aglycones into glycosylated forms of

luteolin (Laguerre et al ., 2009). In this work, oleuropein was not the only predominant form of phenolic extracted but was accompanied with high amounts of luteolin-7-O-glucose (12%-26%), luteolin-4-O-glucoside (11%-12%) and apigenin-7-O-glucoside (4% to 8%). The olive leaves used for this study were collected from the olive oil processing plant; the leaves were green yellowish colour. They could be mature leaves. It may explain the higher amount of glycosylated forms of luteolin (luteolin-7-O-glucose, luteolin-4-O-glucoside and apigenin-7-O-glucose) present in the olive leaf extracts in this study.

### 4.6.5 Comparison of Phenolic Profiles between Three Different Olive Leaf Extracts

The absolute concentration of various phenolic compounds identified in three different olive leaf extracts in Tables 4.4-4.6 was determined by the external standard technique. Phenolic compounds quantification was achieved by the peak area in the chromatograms relative to external standards. Calibration curves were obtained by plotting the peak area of each phenolic compound as a function of its standard concentration. The regression equation and correlation coefficient (see Appendix 4.3) for six major phenolic compounds in the olive leaf extract were 0.99, which indicated there was a good linear relationship between concentration and peak area in the detection range.

Figures 4.10.a & 4.10.b compared the most important phenolic compounds in three olive leaf extracts, which was plotted based on concentrations of each phenolic compound in Tables 4.4-4.6. As can be seen in the Figure 4.10.a, There was no significant difference ($P \geq 0.05$) in oleuropein recovered by 80% methanol (v/v) and 80% ethanol (v/v), but both values were significantly higher ($P \leq 0.05$) than oleuropein in boiling water extracts. Boiling water only recovered 36% oleuropein of that recovered by alcoholic solvent.

The majority of phenolic compounds were present in higher concentration in alcoholic extracts than boiling water extracts as shown in Figure 4.10.a & 4.10.b. When comparing methanol extracts with ethanol extracts, significantly higher concentration ($P \leq 0.05$) of luteolin-7-O-glucosie, tyrosol and hydroxytyrosol were found in methanol extracts than ethanol extracts. Apart from these three compounds, there were no significant differences ($P \geq 0.05$) in concentrations of other major phenolic compounds between methanol extracts and ethanol extracts.

Significantly higher flavonoids were found in alcoholic base extracts than boiling water extracts; apigenin and luteolin were not found in olive boiling water extracts. Instead, relatively higher percentage of phenolic acids such as ferulic acid (1.68%), caffeic acid (0.53%), p-coumaric acids (2.17%) were found in boiling water extracts. The results are in agreement with findings from a previous study completed by Mailk et al. (2008), who reported simply boiling of olive leaf powder in pure water for 10 minutes is quite effective for extraction of oleuropein and verbascoside, but not for luteolin compounds such as luteolin-4-O-glucoside and luteolin-7-O-glucoside, it may be due to degradation of those compounds during boiling (Mailk, 2008).

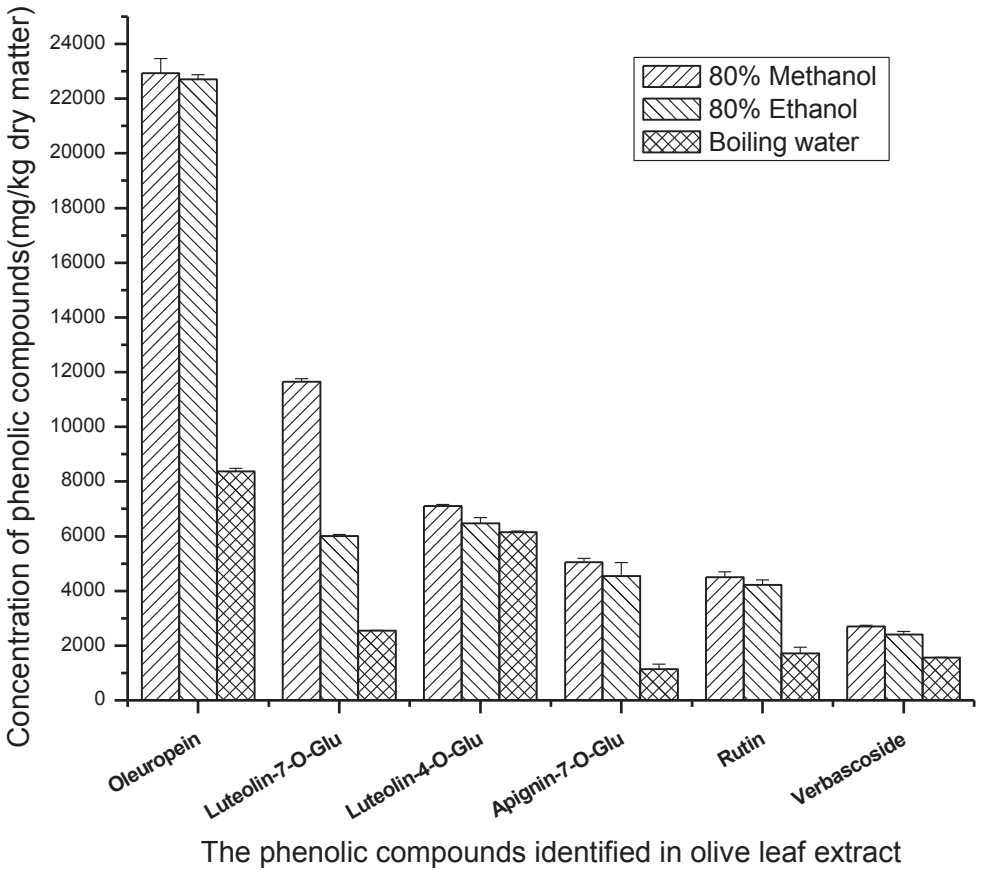



Figure 4. 10.a: Comparison of Phenolic Compounds in Olive Leaf Extracts Using Different Solvents, All Experiments Were Carried out in Triplicates and Data Points Are Mean Value of Triplicates (N=3)

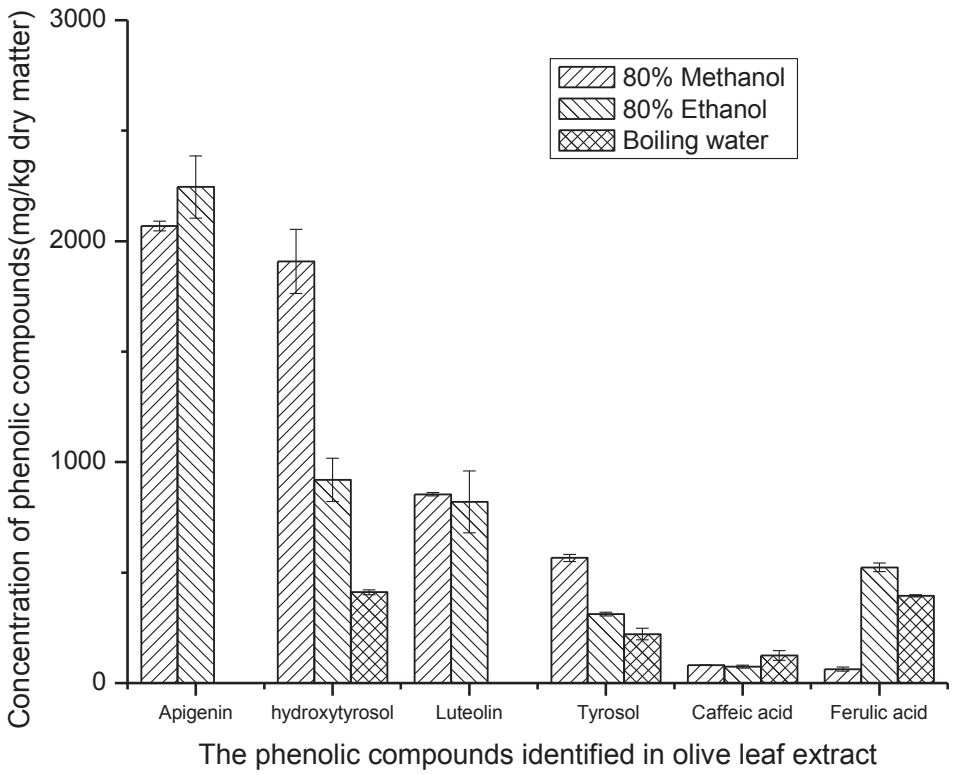



Figure 4.10.b: Comparison of Phenolic Compounds in Olive Leaf Extracts Using Different Solvents, All Experiments Were Carried out in Triplicates and Data Points Are Mean Value Of Triplicates (N=3)

The recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compounds in the solvent used for extraction process. Solvent polarity plays a key role in increasing phenolic solubility. Alcoholic solvent have been commonly used to extract phenolics from natural sources. The most widely used is methanol and methanol/water mixtures. Soluble phenolic compounds are generally extracted using water, methanol, ethanol or acetone. The presence of attached sugars in the structure tends to make the phenolic compounds more water soluble and thus the combinations of the above solvents with water are better for glycosides. Less polar aglycones tend to be more soluble in non-aqueous solvents (Abaza et al., 2011).

Visioli et al. (2002) reported that the olives contain complex phenols, as glycosides, present in a rather polar and hydrophilic form, whereas the oil contains their aglyconic form, which is the most lipid-soluble part of the molecule. Most of phenolic compounds found in olive leaf extracts are glycosides; it may explain that good recovery of those

compounds was achieved using a combination of alcohol and water. Water is the most polar solvent of all, the polarity index of water, methanol and ethanol are 9.0, 5.2 and 5.1 respectively; however least phenolic compounds were obtained in boiling water extracts. Poor recovery of boiling water can be explained by the overheating effect of boiling water (10 minutes), which leads to oleuropein degradation. Also the polarity of water decreases with increasing temperature (Sahin et al., 2011).

## 4.7 The Effect of Cultivar and Leaf Collecting Time on Phenolic Composition of Olive Leaf Extracts

The impact of cultivars and leaf collection time on phenolic composition of olive leaf extracted was evaluated, two cultivars 'Frantoio' and 'Barnea' were investigated, and leaves were collected from their respective tree in October and November 2010. The moisture content and total phenolics of fresh 'Frantoio' and 'Barnea' leaves varied with collection time as shown in Table 4.8; significantly higher value of total phenolic content ($P \leq 0.05$) was observed in 'Barnea' than 'Frantoio' regardless of collection time. The leaves collected in October had significantly higher total phenolics ($P \leq 0.05$) than those collected in November irrespective of cultivars.

Table 4. 8: Moisture Content and Total Phenolic Contents of Fresh Olive Leaf ('Frantoio' Vs. 'Barnea') Collected In Two Different Months

| Cultivars and collect time | Collected in October 2010 | | Collected in November 2010 | |
|---|---|---|---|---|
| | 'Frantoio' | 'Barnea' | 'Frantoio' | 'Barnea' |
| Moisture content (%) | 58.48±0.56 | 62.66±1.71 | 61.60±0.19 | 62.35±1.25 |
| Total phenolic content ( mg caffeic acid / g dry matter) | 17.17±0.12 | 25.17±0.67 | 15.75±0.27 | 17.20±0.58 |

*Data are expressed as mean ±SEM (n=3)

A typical phenolic profile of extracts prepared from fresh leaves of cultivar 'Frantoio' and 'Barnea' collected in November 2010 is shown in Table 4.9. Chromatographic profiles of the different cultivars studied showed no differences in qualitative composition. The main compounds detected by HPLC were identified as oleuropein, rutin, luteolin 7-glucoside, apigenin 7-glucoside and luteolin-4-O-glucoside. Other minor components were: apigenin, hydroxytryrosol, tyrosol, luteolin and verbascoside.

Table 4. 9: Phenolic Composition (mg/Kg Dry Matter) of Fresh Olive Leaf ('Frantoio' vs. 'Barnea'collected) in November 2011

| **Phenolic compounds** | **Cultivars** | |
|---|---|---|
| | **'Frantoio'** | **'Barnea'** |
| Oleuropein | 8030±633 | 8644±393 |
| Luteolin-7-O-glucoside | 5348±21 | 4450±265 |
| Luteolin-4-O-glucoside | 3332±76 | 3417±114 |
| Rutin | 3311±253 | 2620±144 |
| Apignin-7-O-glucoside | 1987±62 | 2386±157 |
| Apigenin | 909±25 | 1296±53 |
| hydroxytyrosol | 716±71 | 321±29 |
| Tyrosol | 593±9 | 548±34 |
| Luteolin | 581±88 | 628±35 |
| Verbascoside | 206±23 | 200±4 |

*Mean ±SME (quantification by external standard technique)

Figure 4.11 compares the concentrations of each phenolic compound in 'Frantoio' and 'Barnea' collected in two different time periods. The olive leaves of the two cultivars showed considerable contents of oleuropein and apignin-7-O-glucoside, which with 'Barnea' was significantly higher ($P \leq 0.05$) than 'Frantoio' for all collected leaves.

Briante et al. (2002) reported that the differences in phenolics compounds of olive leaf samples could be related with the cultivar, collection date of leaves and drying process. It is known that the concentration of phenolic compound in the leaves varies at the time of fruits maturation (Briante et al., 2002). With respect to oleuropein, Ranalli et al. (2006) suggested that the content of oleuropein in the olive leaves was significantly affected by the genetic factor, but barely influenced by the collect period. Its concentration was also markedly affected by the colour and age of leaves, oleuropein was higher in the leaves with an intense green colour (including mainly those at the developing stage).The oleuropein in olive leaves is gradually degraded with their progressive aging, due most probably to biochemical pathways in which the endogenous β-glucosidase enzyme could play a key role (Ranalli et al., 2006)

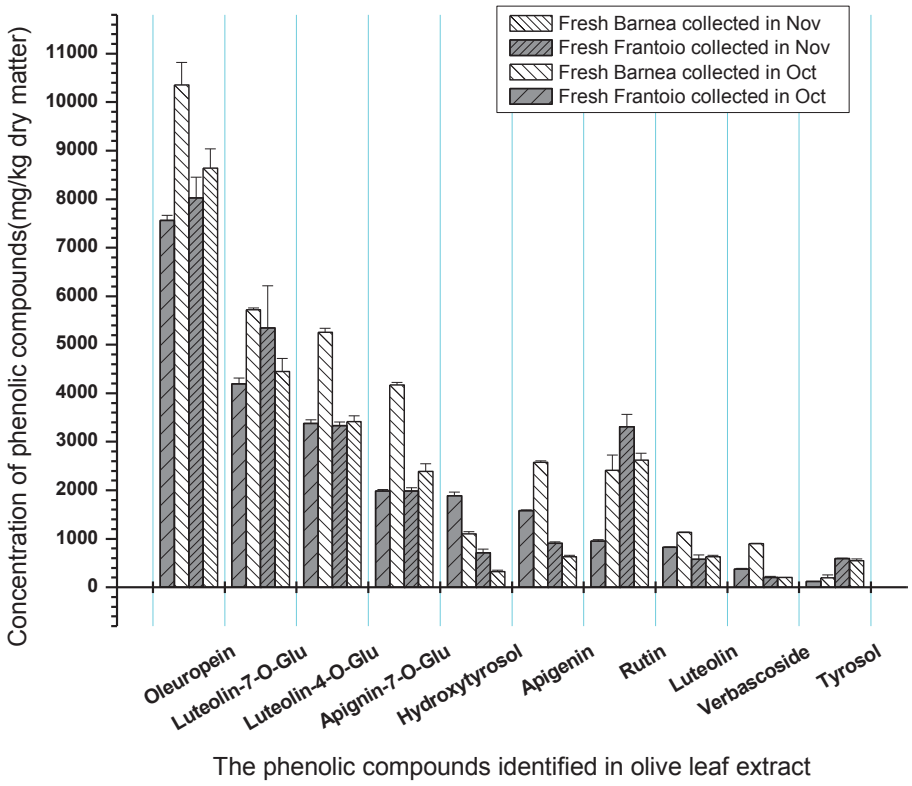



Figure 4. 11: Comparison of Phenolic Composition of Olive Leaves of 'Frantoio'vs. 'Barnea' Collected In October and November 2010, All Experiments Were Carried Out In Triplicates And Data Points Are Mean Value Of Triplicates (N=3)

However, it should be pointed out that the results in this section may not reflect the true pictures of impact of collection time and cultivar on phenolic composition in olive leaves; it cannot be concluded that 'Barnea' contains more phenolic compounds than 'Frantoio', or the phenolic content in the leaves declined from October to November. The leaves used in this section were received from the olive oil plant; one sample was measured in triplicates for each cultivar. Despite standard error of three measurements showed good repeatability, however values may not represent the true value of the population. There was no information about the ages and colour of leaves; hence the results in this section are inconclusive.

In order to identify the impact of cultivar and collection time on the oleuropein level of leaves, further investigation needs to be carried out. The harvest season for 'Frantoio' and 'Barnea' olive fruits are around June and July in Hawks Bay in New Zealand, and thus leaf

samples may need to be collected throughout year and the colour of leaves will also need to be monitored and green leaves would be preferable for further study.

## 4.8 The Effect of Drying On Phenolic Compounds of Olive Leaf Extract

The effect of drying on total phenolics and individual compounds of 'Frantoio' olive leaves is shown in Table 4.10 and Figure 4.12. The total phenolics in dried leaves were significantly higher than in fresh leaves ($P \leq 0.05$). The concentration of individual phenolic compounds was also significantly higher ($P \leq 0.05$) in dried leaves than fresh leaves except tryosol and hydroxytyrosol, which was found to be higher in fresh leaves than dried leaves. Microwave oven dried olive leaves were found to contain the highest values of oleuropein, luteolin-7-O-glucoside and luteolin-4-O-glucoside. Twenty percent more oleuropein was recovered in microwave oven dried leaves than air oven dried leaves. The chromatograms of three different samples are given in Appendix 4.4; the difference in peak intensity of individual phenolic compounds including oleuropein (peak 6), luteolin-7-O-glucoside (peak 5) and luteolin-4-O-glucoside (peak 7) between three samples could be easily identified in the graph.

The results in this study show that the oleuropein content of olive leaves was significantly ($P \leq 0.05$) influenced by drying process. Drying increased the level of most of phenolics compounds, especially oleuropein. Dried olive leaf contains nearly double amounts of oleuropein than fresh leaves. Microwave oven drying was better than air oven drying in terms of total phenolic compounds recovered, despite air oven drying allows the better retention of the green colour of the fresh leaves than microwave oven drying as shown in Appendix 4.4.

Table 4. 10: Total Phenolic Content (mg Caffeic Acid /g Dry Matter) of Fresh Olive Leaf, Microwave Oven Dried Leaves and Air Oven Dried Leaves

| **Olive leaf sample ('Frantoio' cultivar)** | **Fresh olive leaves** | **Microwave oven dried olive leaves** | **Air oven dried olive leaves** |
|---|---|---|---|
| Total phenolic content (mg caffeic acid /g dry matter) | 15.75±0.27 | 22.37±0.39 | 18.05±0.25 |
| Moisture content (%) | 58.48±0.56 | 7.60±0.12 | 8.44±0.05 |

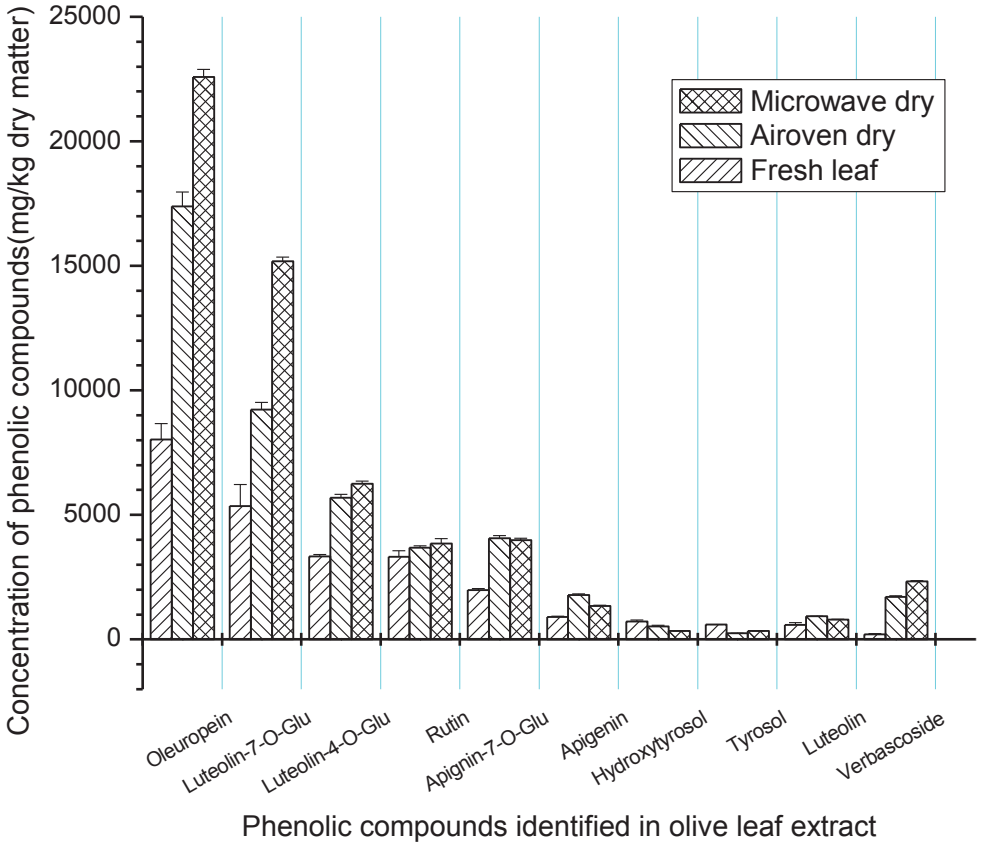



Figure 4. 12: Comparison of Phenolic Composition between Fresh, Microwave Dried and Air Oven Dried Olive Leaves, All Experiments Were Carried out in Triplicates and Data Points Are Mean Value of Triplicates (N=3)

For olive fruits, the rapid decline of phenolic content that occurs during the black maturation phase is most likely correlated with the increased activity of hydrolytic enzymes (Amiot et al., 1989). Malik & Bradford (2006) suggested that the compartmentalization perhaps is the reason for maintaining high oleuropein level in immature green fruits that decline dramatically when fruits turn black and softer, possibly due to leakage of oleuroepin degradation enzyme during softening of olive fruits at maturity (Malik & Bradford 2006). Those hydrolytic enzyme and oleuropein degradation enzyme are β-glucosidase and polyphenol oxidase. Oleuropein is stable when separated from active enzyme such as β-glucosidase and polyphenol oxidase; when oleuropein and these enzymes make contact, oleuropein is broken down and converted into other compounds (Ortega-Garcia., 2008).

Beta-glucosidase belongings to the glucohydrolase enzyme family, which cleaves β-glucosidic linkages in disaccharide or glucose substituted molecules. Beta-glucosidase

occurs in all kingdoms of life and play fundamental biological roles in processes such as degradation of cellulose and other carbohydrates for nutrient uptake and developmental regulation or chemical defense against pathogen attack (Jeng et al., 2011). In addition, beta-glycosidase plays a key role in aroma formation in tea, wine, fruit juice and vanilla. The characteristic aroma of vanilla is only obtained after a curing process, which hydrolyse the glucosides and to release the aroma compounds and β-glucosidases are believed to play an important role in this process (Arana, 1943).

*O.europaea* tissues contain large amount of β-glucosidase which specifically hydrolyses oleuropein. Oleuropein and β-glucosidase located in the different cell compartments of fruits, oleuropein is contained in the vacuoles of olive mesocarp cells, and β-glucosidase is in the mesocarp cell chloroplasts. The damage of cells and tissues bring in contact β-glucosidase with oleuropein with consequent decrease of oleuropein to form oleuropein aglycone and glucose (Mazzuca et al., 2006). The involvement of β-glucosidase in the progressive degradation of oleuropein and the release of glucose and aglycone during fruit ripening at different stages of fruit ripening are strictly related to olive oil quality. Oleuropein aglycone contains a hydroxytrosol group which is antioxidant moiety. The olive oil also contains hydroxytrosol that is mainly formed from the hydrolysis of the oleuropein aglycone, but in small amounts than aglycone.

For olive leaf, results was observed when freezing and thawing fresh olive leaf caused a sharp decline of oleuropein due to breakage of plant cell membrane by ice crystal in fresh leaves, resulting in release of oleuropein degrading enzyme (Malik et al., 2008). Significantly less oleuropein ($P \leq 0.05$) was recovered from fresh leaves than dried leaves is possibly due to mixing of oleuropein with oleuropein degrading enzyme that are compartmentalised in fresh leaf cells. Fresh leaves were ground in coffee grinder before extraction in this study, which may result in breakage of cell membranes and release of the oleuropein degrading enzyme. The energy produced by powdering process may activate the enzyme. High water content in fresh olive leaf (Table 4.10) accelerates the hydrolysis of oleuropein. Degradation of luteolin glycosides in fresh olive leaves was much slower than oleuropein, which may indicate the enzyme responsible for their degradation was not very active under same condition.

Malik & Bradford (2008) reported that air drying at 25°C or elevated temperature such as 30 °C and 40°C in the oven results in good recovery of oleuropein, however, drying at 60°C would result in substantial losses of polyphenols possibly due to degradation of oleuropein and other polyphenols (Malik & Bradford 2006).

In this study, intact olive leaves were dried at 30°C for 48 hours, most of water was removed before breakage of cell membrane during grinding; there was only 7-8% of water content remaining in the dired leaves (Table 4.10). The degradation of oleuropein may also take place during drying as there is still water and oxygen present. However the degradation appeared to be much less than in fresh leave. This is possible due to temperature used for drying in this study under which β-glucosidase may still be inactive.

Savournin et al. (2001) suggested that microwave drying avoided ester hydrolysis of saponins, which occurred with air-drying; higher amount of oleuropein was obtained in microwave oven dried olive leaves (Savournin et al., 2001). Microwave drying has the advantage of much shorter drying time so that it could avoid leaves being exposed for a long time to light and high temperature, which may lead to oxidation of some phenolic compounds due to enzymatic activity.

On the other hand, drying processes caused changes in the physical and structural proprieties of olive leaves and it might also have significant influence on the solvent extractability of phenolic compounds as the ability of solvent permeation and diffusion into and from ground powdered leaves between fresh and dried leaves can be difference.

Oven-drying and air-drying at ambient temperatures are conventional sample pre-treatment for phytomaterials (Obied et al., 2008). The majority of studies on olive leaf phenolics have been carried out previously by using dried olive leaf and different drying technique was applied such as air oven drying, freezing drying, and infrared drying. Of those drying methods, air oven drying was mostly adopted by a number of researchers (Le et al., 1998; Savournin et al., 2001; Bouaziz & Sayadi 2005; Japon et al., 2006; Malik & Bradford 2006). Drying could also be a good way to preserve fresh olive leaves. Their high moisture content (see Table 4.8) would easily lead microbial spoilage of olive leave.

## 4.9 Investigation of the Stability of Olive Leaf Extract

### 4.9.1 The Changes in Total Phenolics of 60% and 80% ethanol olive leaf extracts during storage

Olive leaf extracts were prepared using 60% and 80% aqueous ethanol and then concentrated to10 fold (see Section 3.8.1). The total phenolic and oleuropein concentration of original leaf extracts and concentrated extracts were measured. A mass balance was carried out for the two runs and the results are presented in Appendix 4.5. Minor loss of total phenolic contents (3.18%) occurred during the concentration process.

Figures 4.13 shows the changes in total phenolics and oleuropein concentration of 60% ethanol based olive leaf extracts during storage at four temperatures (-20, 4, 20, 30°C) for 10 to 12 weeks. Two samples t-test at 95% confidence level was conducted to compare if there was any difference between samples before and after storage (see Appendix 4.6). The results show here was no significant change in total phenolics when stored at -20°C ($P \geq 0.05$). At storage of temperature of 4°C, the total phenolic content decreased significantly by 16% after 10 weeks storage ($P \leq 0.05$). The significant loss in total phenolic content was observed at temperatures of $20^0$C and $30^0$C, the total phenolics decreased by 30% and 38% respectively after 12 weeks storage ($P \leq 0.05$). Variability at week 0 was due to differences in the different batches of extract recovered.

The variation of total phenolic content of 80% ethanol based olive leaf extract during storage at four temperatures is shown in Figure 4.14. Similar results as above were obtained from a two sample T-test at 95% confidence level which compared the difference between samples at the beginning and the end of storage (see Appendix 4.7). The results indicate that there was no significant difference in terms of total phenolic when samples were stored at -20°C ($P \leq 0.05$) and 4°C ($P \leq 0.05$) for 10 weeks, however it changed significantly when samples were stored at 20°C and 30°C ($P \leq 0.05$). The total phenolics decreased by 26% and 30% after 12 weeks respectively when stored at these two temperatures.

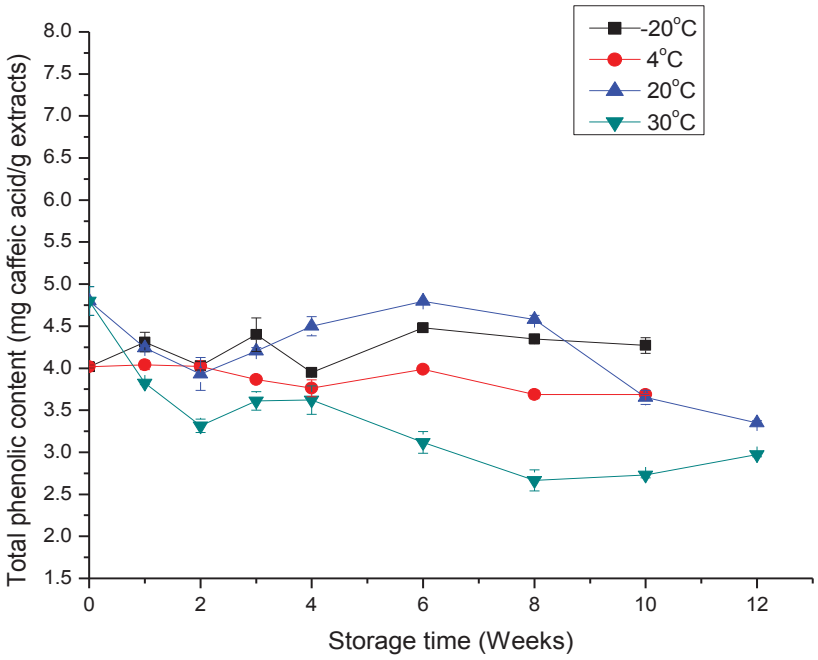



Figure 4. 13: The Changes of Total Phenolic Content of 60% Ethanol Based Olive Leaf Extracts vs. Storage Time at Four Temperatures. All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

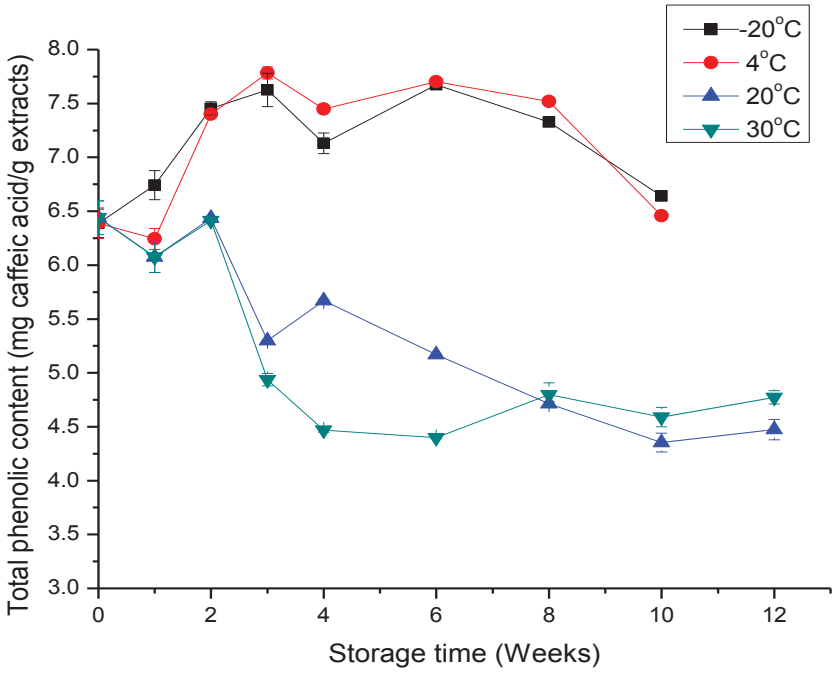



Figure 4. 14: The Changes of Total Phenolic Content of 80% Ethanol Based Olive Leaf Extracts vs. Storage Time at Four Temperatures. All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

### 4.9.2 The Changes In Oleuropein Concentration of 60% And 80% Ethanol Olive Leaf Extracts During Storage

In terms of oleuropein (Figure 4.15), there were no significant changes for 60% ethanol olive leaf extracts (P≥0.02) after 10 weeks storage at -20°C. However, oleuropein decreased significantly when stored at temperatures of 4°C, 20$^0$C and 30$^0$C (P≤0.02). When stored at of 4°C and 20$^0$C, 20% and 26% of oleuropein was lost respectively at the end of storage. At a temperature of 30$^0$C, oleuropein decreased steadily at the beginning of the storage period and then decreased rapidly after week 6, there was 60% loss of oleuropein after 12 weeks storage.

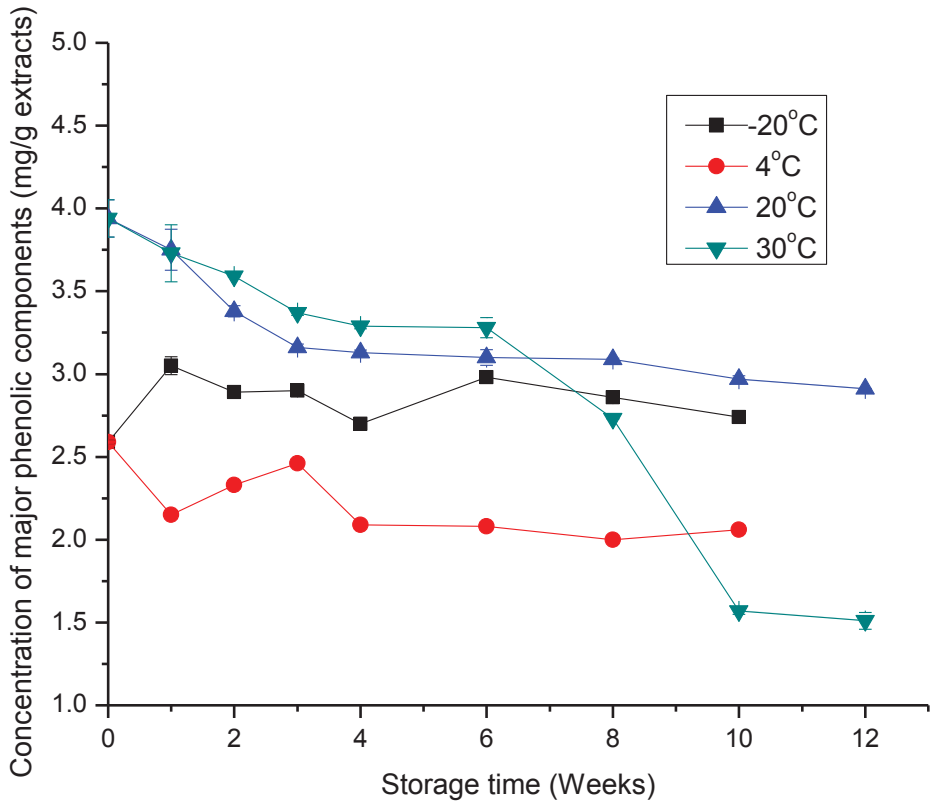



Figure 4. 15: The Changes of The Concentration of Oleuropein of 60% Ethanol Based Olive Leaf Extract vs. Storage Time at Four Temperatures, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

The oleuropein level in 80% olive leaf extracts remained relatively stable when stored samples at -20°C (P≥0.02) as shown in Figure 4.16, while considerable changes were

observed at 4°C (P≤ 0.02), 20$^0$C and 30$^0$C ( P≤ 0.02). The oleuropein decreased by 18%, 28% and 30% respectively at these three storage temperatures.

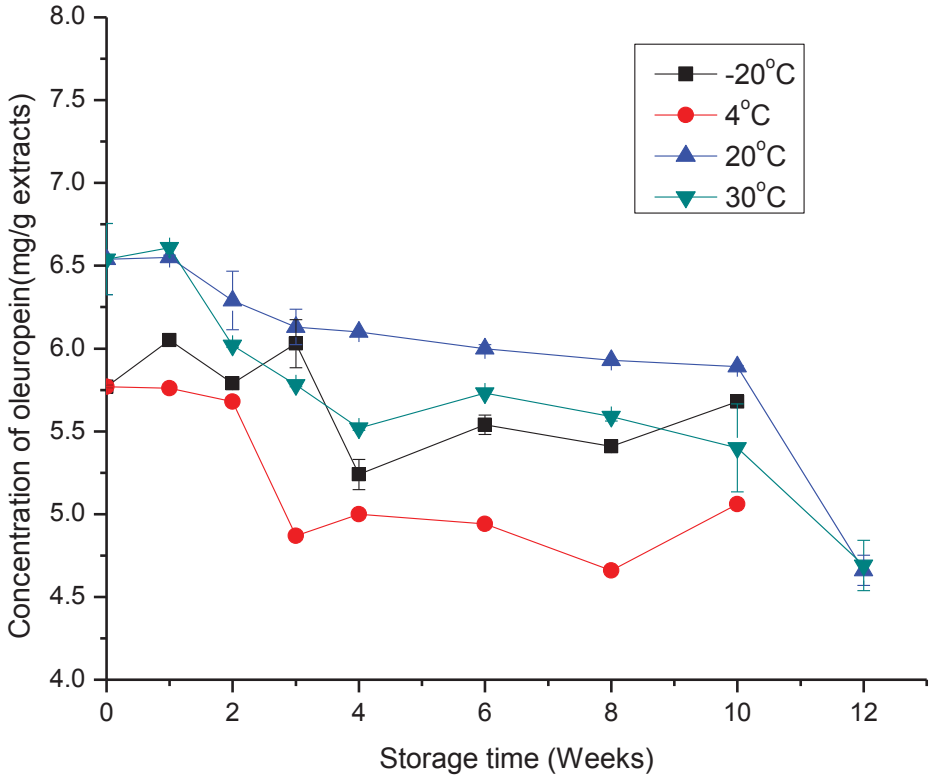



Figure 4. 16: The Changes of The Concentration of Oleuropein of 80% Ethanol Based Olive Leaf Extract vs. Storage Time at Four Temperatures, All Experiments Were Carried out in Duplicates And Data Points Are Mean ±SEM (N=2)

### 4.9.3 The Changes In Antioxidant Activity Of 60% And 80% Ethanol Olive Leaf Extracts During Storage

Antioxidant capacity is a measure of the amount of a given free radical scavenged by an antioxidant (Macdonald-Wicks et al., 2006). Antioxidant capacity is strongly associated with the effectiveness of an antioxidant in the function of disease prevention. Antioxidant capacity assays can be classified into hydrogen atom transfer (HAT) based assays and single electron transfer (ET) based assays as discussed previously in literature review (Huang et al., 2005; Prior et al., 2005). In this study, two assays were conducted to evaluate the changes of antioxidant capacity of olive leaf extracts over storage time; they were 2, 2-Diphenyl-l-picrylhydrazyl (DPPH$^{\cdot}$) free radical scavenging activity assay and

oxygen radical absorbance capacity (ORAC) assay. DPPH method is an easy and accurate ET based method with regard to measuring the antioxidant capacity of fruit and vegetable extracts (Sanchez-Moreno, 2002). The antioxidant capacity is expressed as EC 50 which is defined as the gram of concentrated olive leaf extract necessary to decrease the initial DPPH free radical concentration by 50%, the smaller the EC 50 value is, the more efficient the olive leaf extract is (Brandwilliams et al., 1995). ORAC represents a hydrogen atom transfer HAT reaction mechanism, which is most relevant to human biology (Prior et al., 2005). ORAC values are expressed as micromoles of Trolox equivalent (TE) per gram of olive leaf extract, the higher value, the more efficient the olive leaf extract is.

Figure 4.17 and 4.18 show the variations of antioxidant capacity of 60% ethanol based olive leaf extract measured by DPPH and ORAC during storage. As can be seen in the graphs, antioxidant capacity show a clear decline for the olive leaf extracts stored at all temperatures. The TE value decreased with storage time, while EC 50 increased. The most decrease in TE was observed when the extract was stored at $30^0$C for a period of 12 weeks; the olive leaf extract lost nearly half of its antioxidant capacity. In respect to the EC 50 value, similar results were obtained at $30^0$C, the EC 50 value increased by 50% at the end of storage indicating a loss of phenolic compound and scavenging ability. The least loss of antioxidant capacity was observed when extracts were stored at -20°C.

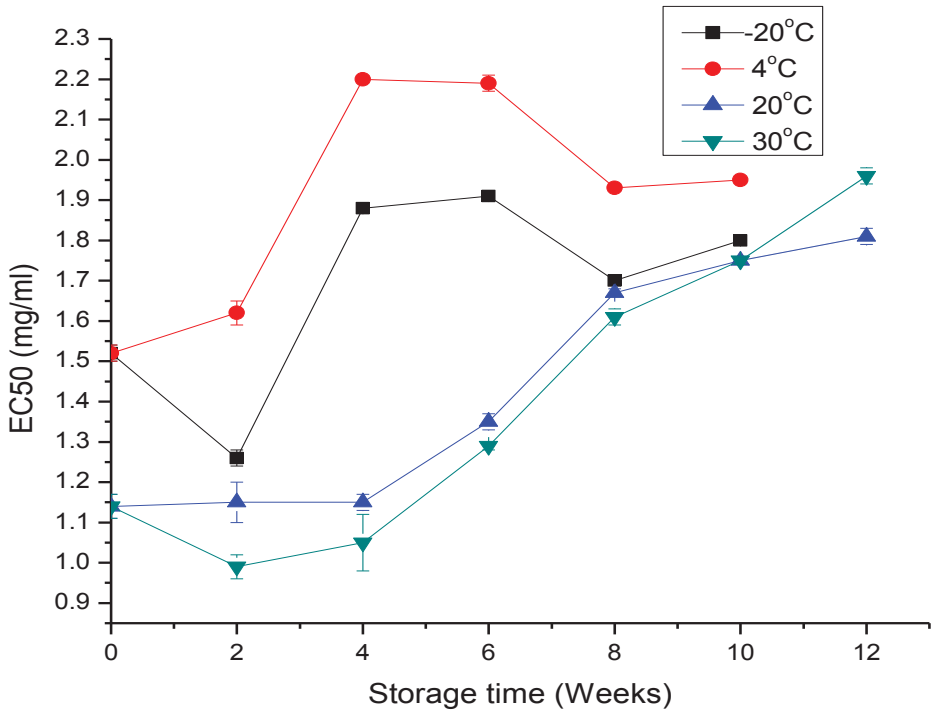



Figure 4. 17: The Changes of The DPPH Free Radical Scavenging Activity (DPPH) of 60% Ethanol Based Olive Leaf Extract vs. Storage Time at Four Temperatures All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

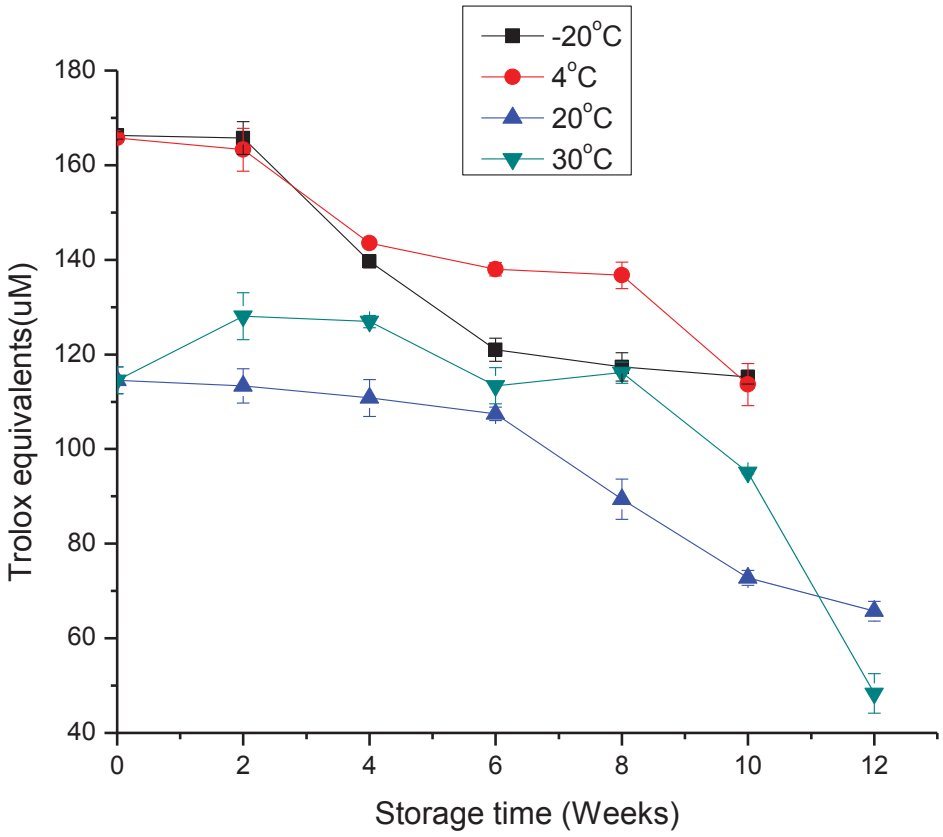



Figure 4. 18: The Changes of The Oxygen Radical Absorbance Capacity (ORAC) of 60% Ethanol Based Olive Leaf Extract vs. Storage Time at Four Temperatures All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

With regard to antioxidant activity of 80% ethanol leaf extracts, a decrease in TE value and increase in EC50 were observed when stored at all four temperature for 10 to 12 weeks(see Figure 4.19 & 4.20). Samples stored at 30°C lost the most antioxidant capacity, TE value of olive leaf extract decreased by 42% and EC50 value decrease by 56% (EC50) respectively. There was less loss of antioxidant capacity when samples were stored at -20°C. At a storage temperature of 4°C, the loss of antioxidant capacity was approximately 30% in terms of both TE and EC50 values. The decrease in antioxidant capacity may be due to the loss of important phenolic compounds during storage such as oleuropein, which has a high antioxidant capacity with high free radical scavenging activity (Visioli et al., 2002).

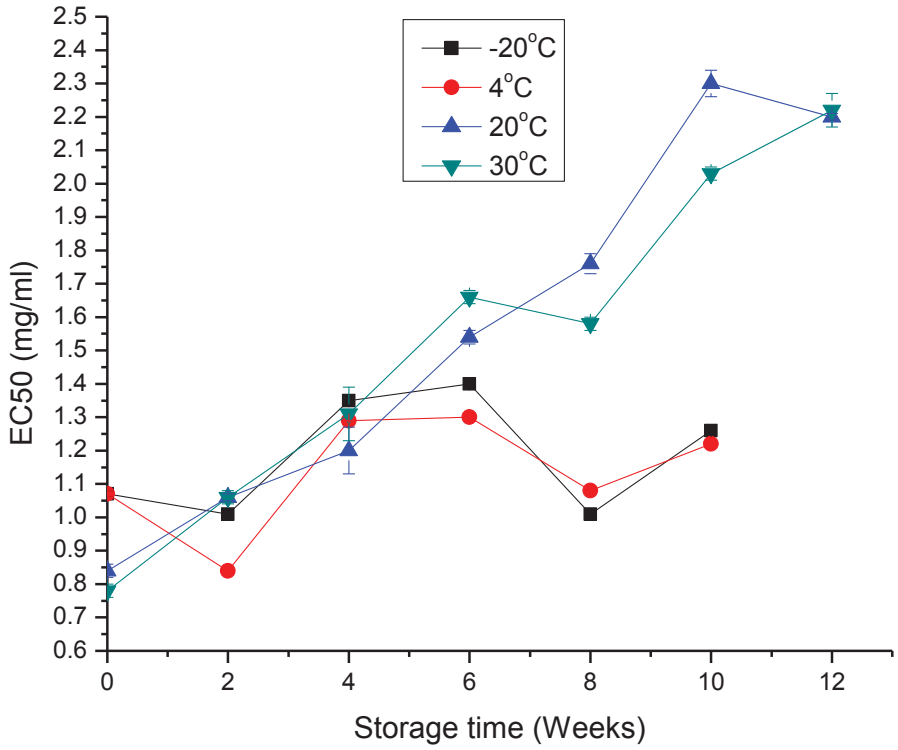



Figure 4. 19: The Changes of The DPPH Free Radical Scavenging Activity (DPPH) of 80% Ethanol Based Olive Leaf Extract vs. Storage Time at Four Temperatures,All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

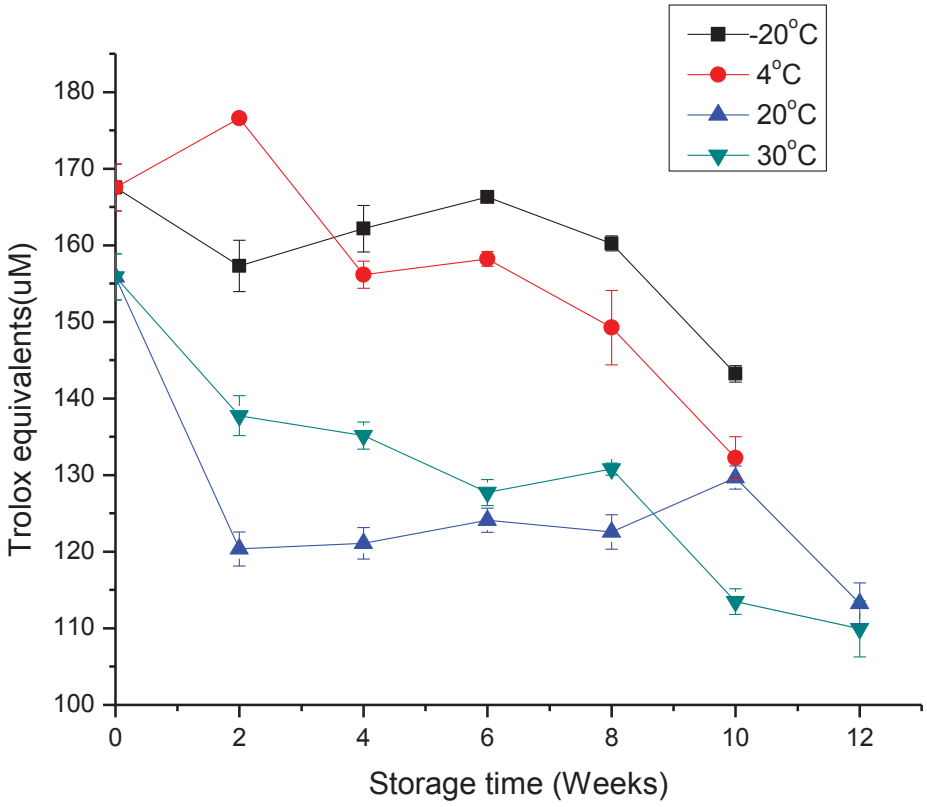



Figure 4. 20: The Changes of The Oxygen Radical Absorbance Capacity (ORAC) of 80% Ethanol Based Olive Leaf Extract vs. Storage Time at Four Temperatures,All Experiments Were Carried out in Triplicates and Data Points Are Mean ±SEM (N=2)

### 4.9.4 The Reaction Kinetics For Degradation Of Phenolic Compounds In Olive Leaf Extracts

The rate of degradation and the order of reaction for the phenolic compounds were evaluated; the detailed calculations are given in Appendix 4.8. Degradation of total phenolics and oleuropein was assumed to follow zero order. Storage temperature also has an impact on the degradation of total phenolic content and oleuropein, and higher temperature may result in faster degradation of phenolic compounds including oleuropein. To estimate the temperature effect on the rate of degradation, activation energy ($E_A$) was calculated by the Arrhenius equation. The activation energy for degradation of oleuropein of 60% and 80% ethanol olive leaf extracts were 9.004 J/(mol K) and 41.99 J/(mol K) respectively.The result shows that olive leaf extract degradation is sensitive to temperature and ethanol concentration.

### 4.9.5 The Changes of Phenolic Composition of 60% and 80% Ethanol Based Olive Leaf Extracts during Storage

The variation of major phenolic components in the 60% ethanol based olive leaf extracts during storage was measured by HPLC (see Section 3.3). Figures 4.21-4.24 show the changes in concentrations of important phenolic compounds when stored at the four storage temperatures. The phenolic composition remained relatively stable in all the samples were stored at -20°C, whereas hydroxytyrosol, apigenin increased slightly by10-16% when stored samples at 4°C. Two sample t-test at 95% confidence level for samples stored at 20°C and 30°C showed that there were significant increases in hydroxytyrosol, apigenin and luteolin ($P \leq 0.05$)) at these two temperatures , along with a decrease in oleuropein, apigenin-7-glucoside and luteolin-4-O-glucoside. Hydroxytyrosol, apigenin and luteolin increased by 60%, 23% and 40% by week six, and then continued to increase, the final concentration of hydroxytyrosol, apigenin and luteolin doubled at the end of storage. The concentrations of oleuropein, luteolin-4-O-glucoside and apigenin-7-O-glucoside decreased by 26% to 30% when samples were stored at 20°C. At a temperature of 30°C, hydroxytyrosol and apigenin doubled and luteolin increased by 40% at the end of week six, whereas the concentration of oleuropein, luteolin-4-glucoside and apigenin-7-glucoside decreased by 40% to 60%. The final concentration of hydroxytyrosol was three times greater than the original amount at the end of storage.

Hydroxytyrosol, apigenin, caffeic acid and luteolin are breakdown products of oleuropein, luteolin-4-O-glucoside, verbascoside and apigenin-7-O-glucoside. The degradation of these compounds was also observed in dried olive leaves (Tsimidou & Papoti, 2010). All concentration changes of major phenolic compounds contribute to the change of total phenolic contents in olive leaf extracts.

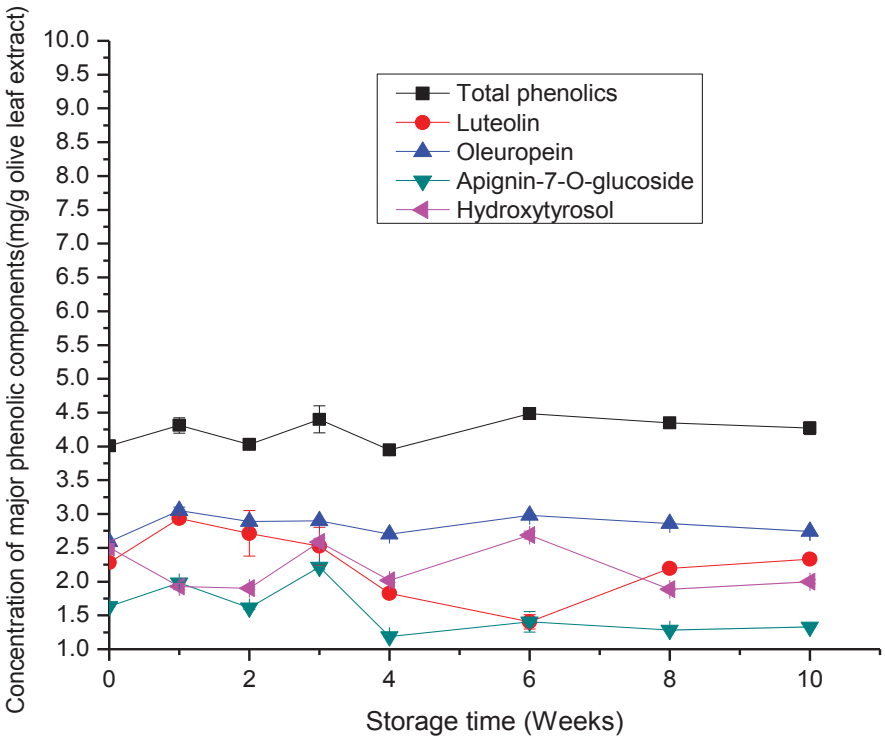



Figure 4. 21a: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at -20°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

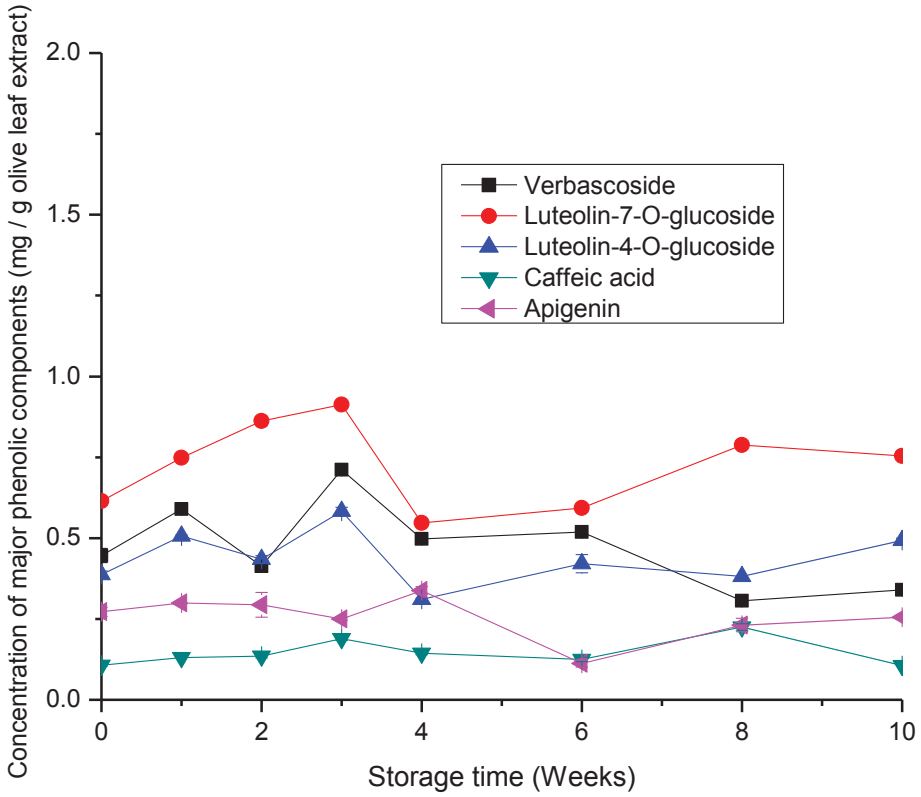



Figure 4.21.b: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at -20$^0$C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

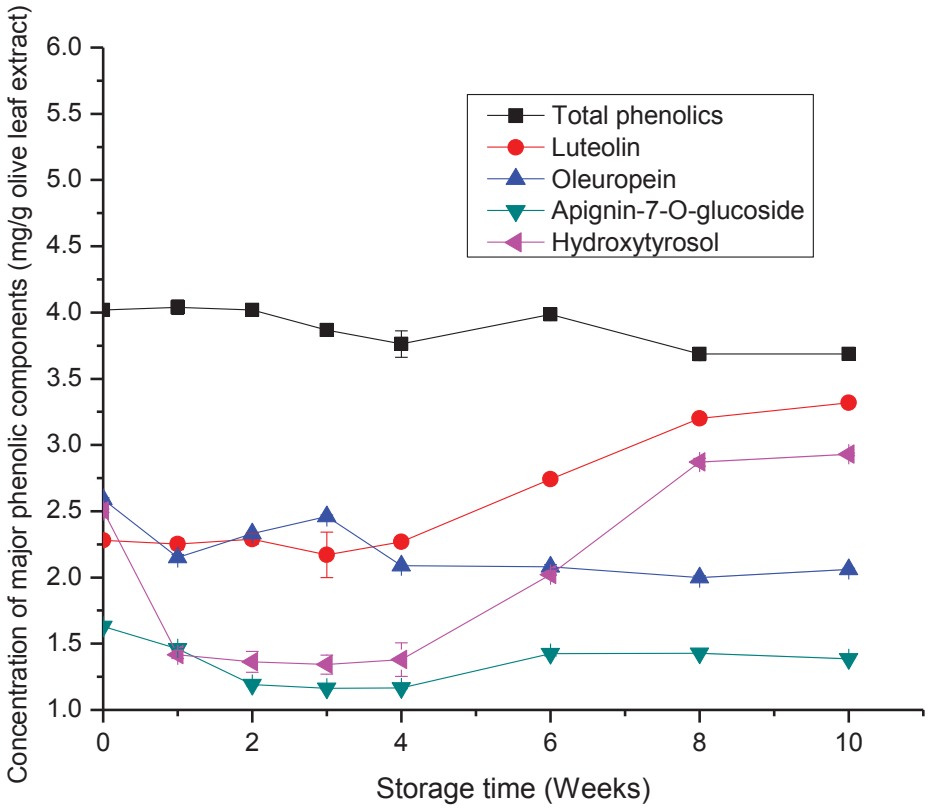



Figure 4. 22.a: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at 4°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

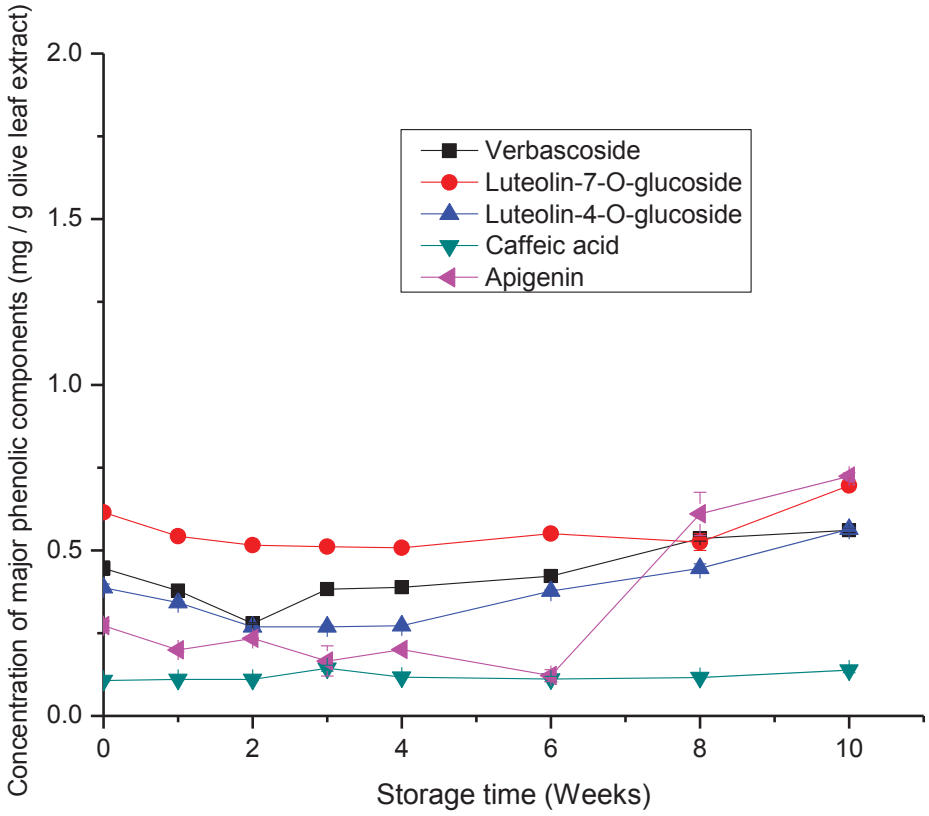



Figure 4.22.b: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at 4°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

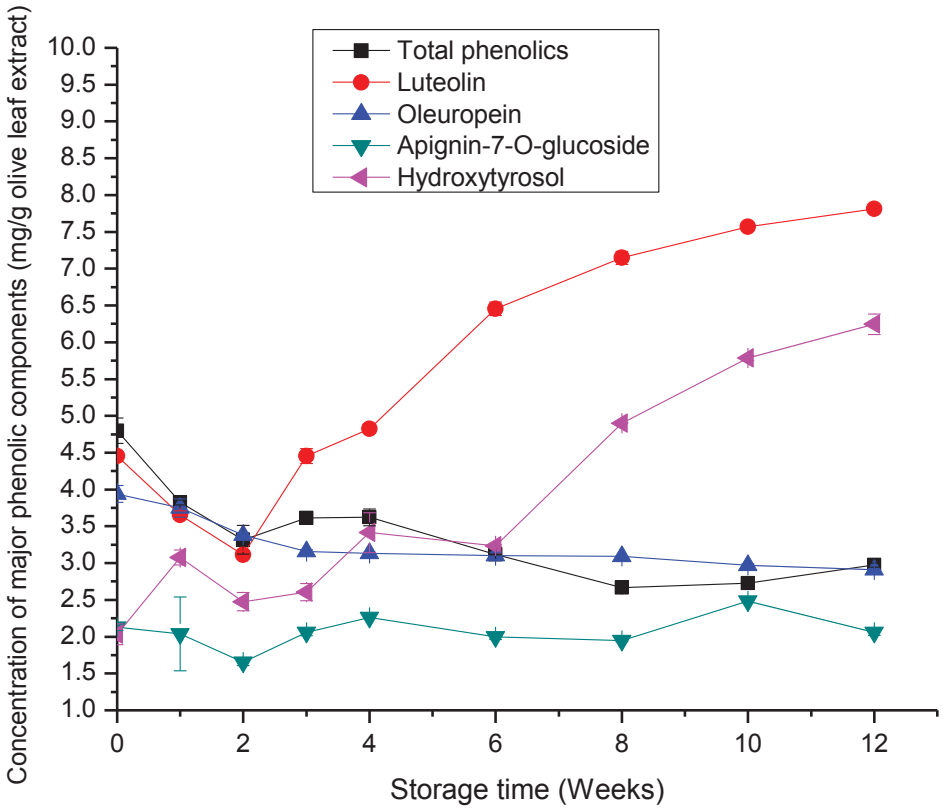



Figure 4. 23.a: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at 20°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

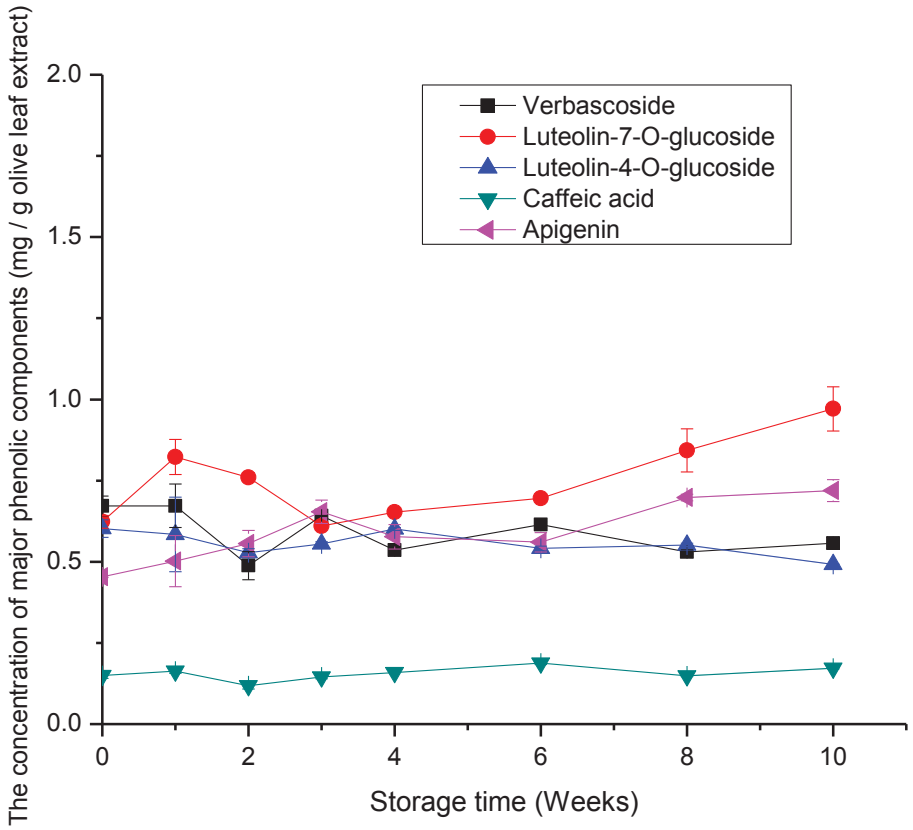



Figure 4.23.b: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at 20°C. All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

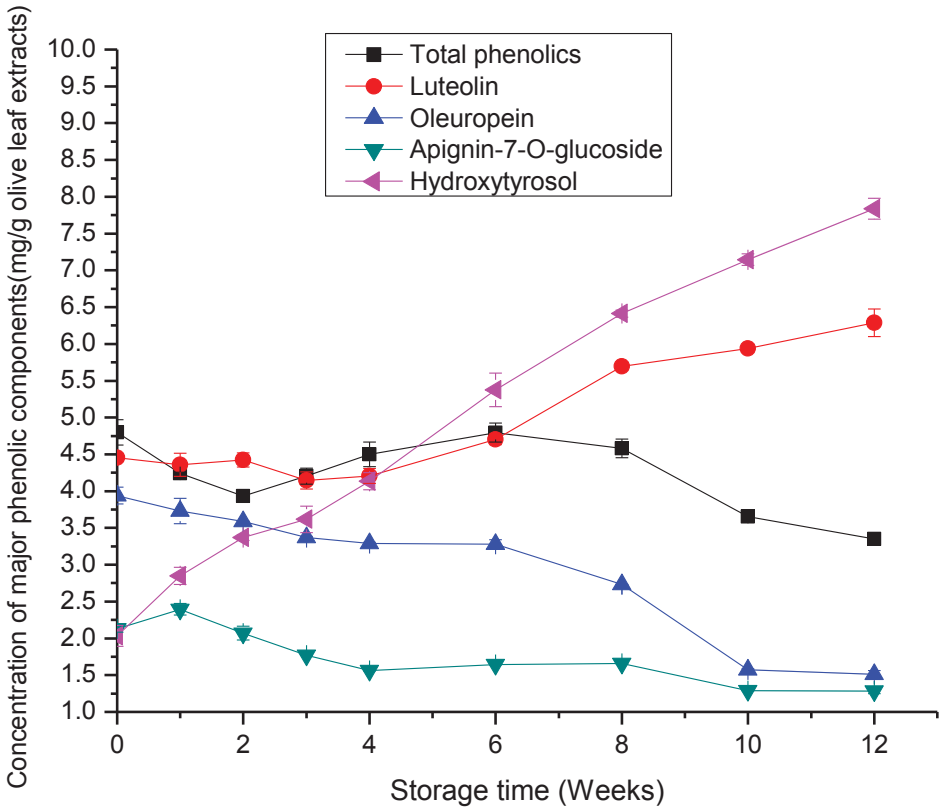



Figure 4. 24.a: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at 30°C.All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

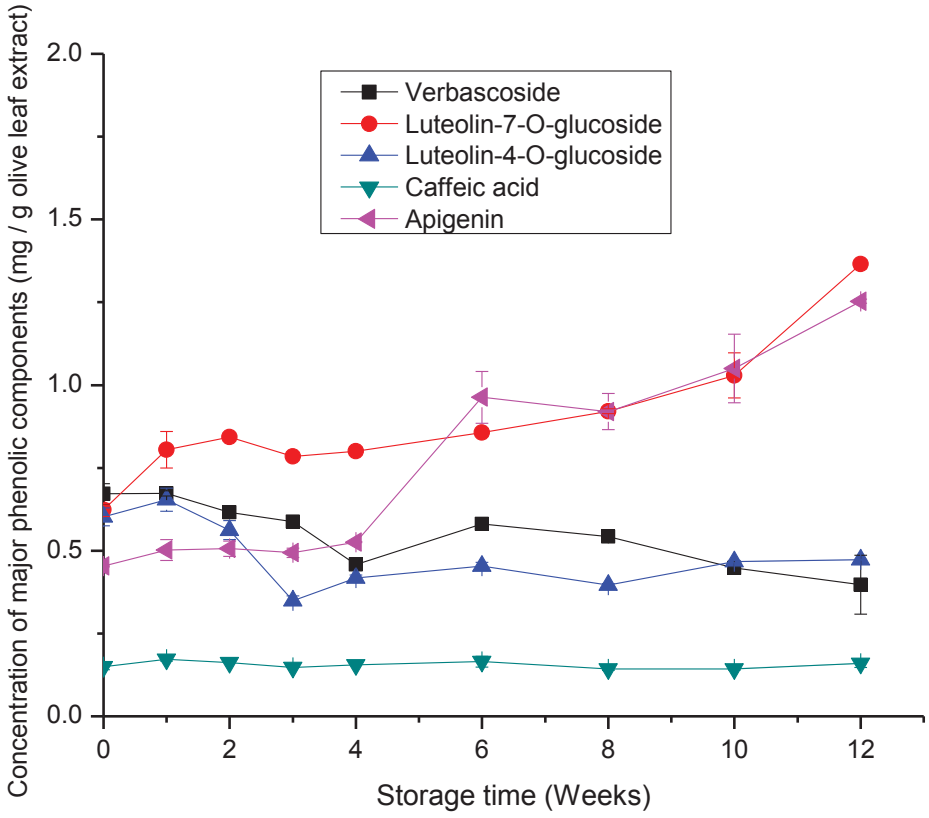



Figure 4.24.b: The Concentration Change of Major Phenolic Components of 60% Ethanol Based Olive Leaf Extract When Stored at 30°C.All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

The changes of phenolic composition of 80% ethanol based olive leaf extracts during storage are shown in Figure 4.25-4.28. The concentration of all important phenolic compounds remained relatively stable when kept at -20$^0$C after 10 weeks storage. Hydroxytyrosol, apigenin increased by10-40% when samples were stored at 4 $^0$C, along with 10-20% of decreasing in oleuropein and apignin-7-O-glucoside, while luteolin and luteolin-4-O-glucoside remained almost constant. At a temperature of 20 °C, the concentration of hydroxytyrosol, apigenin and luteolin increased by 70% to 90% at the end of storage, and dramatic changes of those compounds were observed after week 6. Approximately 25% to 30% of oleuropein, luteolin-4-O-glucoside and apigenin-7-O-glucoside were lost when stored at this temperature. Hydroxytyrosol and luteolin doubled in concentration and apigenin increased by 70% when olive leaf extracts were stored at

30 °C for 12 weeks, whereas the concentration of oleuropein, luteolin-4-glucoside and apigenin-7-O-glucoside decreased by 20% to 40%.

In summary, the concentration of oleuropein, apigenin-7-O-glucoside and luteolin-4-O-glucoside in the concentrated olive leaf extract, decreased with increasing storage time, whereas those of their respective metabolites (hydroxytyrosol, apigenin, luteolin) exhibited the opposite trend. The effect of temperature on the storage time increased with increasing temperature from 4°C to 30°C, the phenolic composition remained relatively constant in the sample kept at -20°C. Caffeic acid is a breakdown product of verbascoside, however, the concentration of verbascoside and caffeic acid remained relatively stable at all four temperatures studied. The concentration of luteolin-7-O-glucoside was found to increase with increasing storage time and temperature. The possible reason could be luteolin-7-O-glucoside co-eluted with rutin during the HPLC analysis, and thus the concentration of luteolin-7-O-glucoside may have been overestimated. Though, the result suggests that there was degradation of flavonoids in stored samples at higher temperatures. Overall, the results suggest that less than 5% of phenolic compounds and antioxidant capacity was lost when olive leaf extracts were stored at -20°C. Increasing temperature caused degradation of important phenolics including oleuropein. The greater extent of oleuropein degradation was observed in 60% ethanol olive leaf extracts. The degradation of olive leaf extracts stored at higher temperatures appears to increase from week 6. It is also suggested that storage at -20 °C was suitable for the storage of olive mill waste for 12 months (Obied et al., 2005).

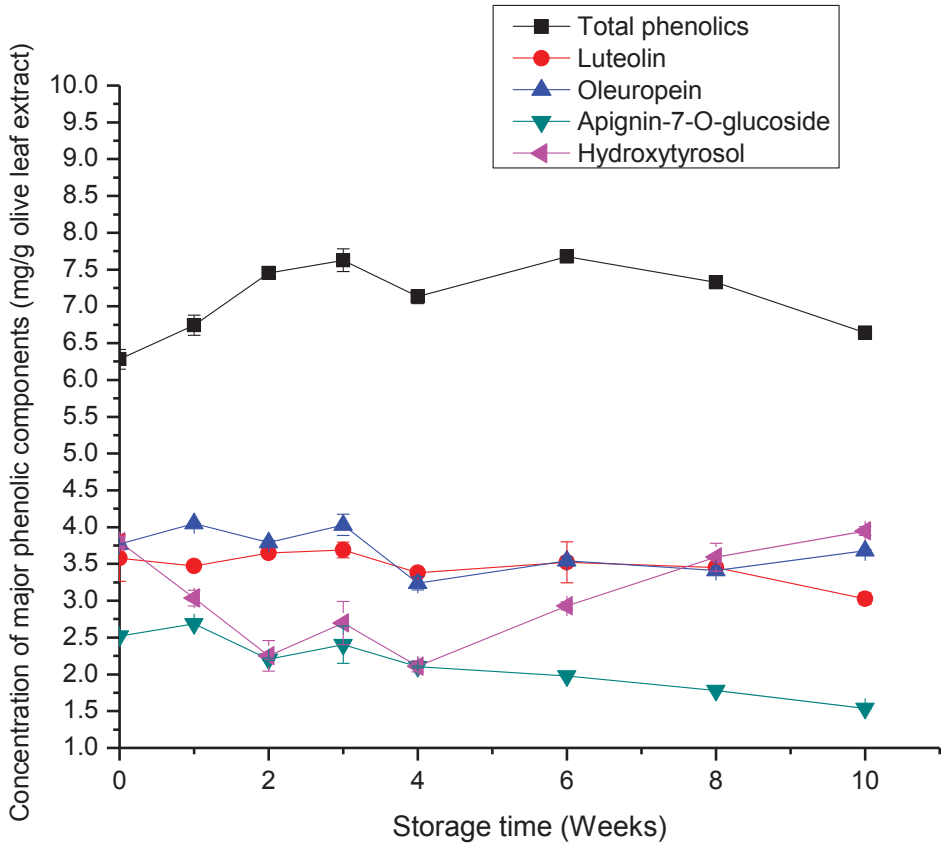



Figure 4. 25.a: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at -20°C.All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

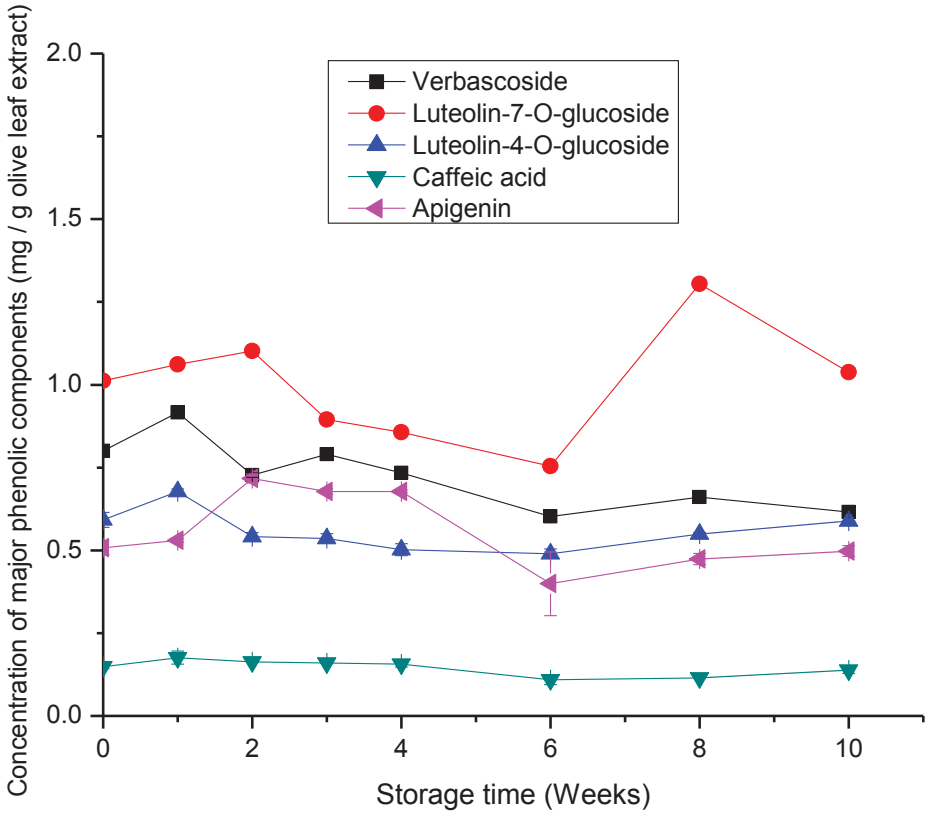



Figure 4.25.b: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at -20°C.All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

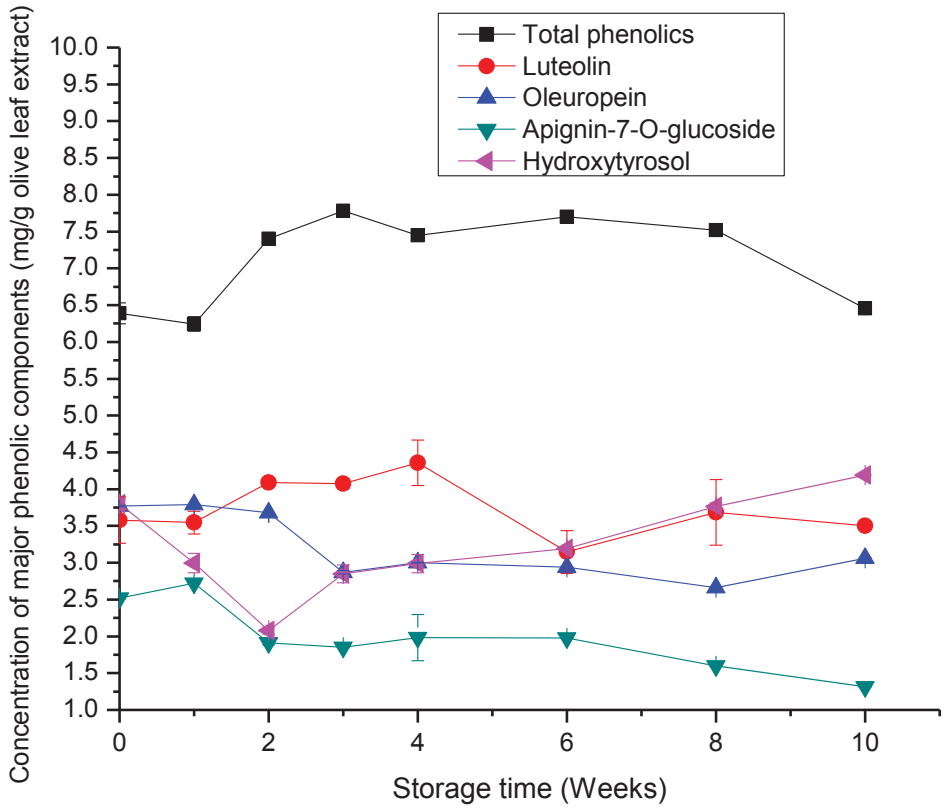



Figure 4. 26.a: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at 4°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

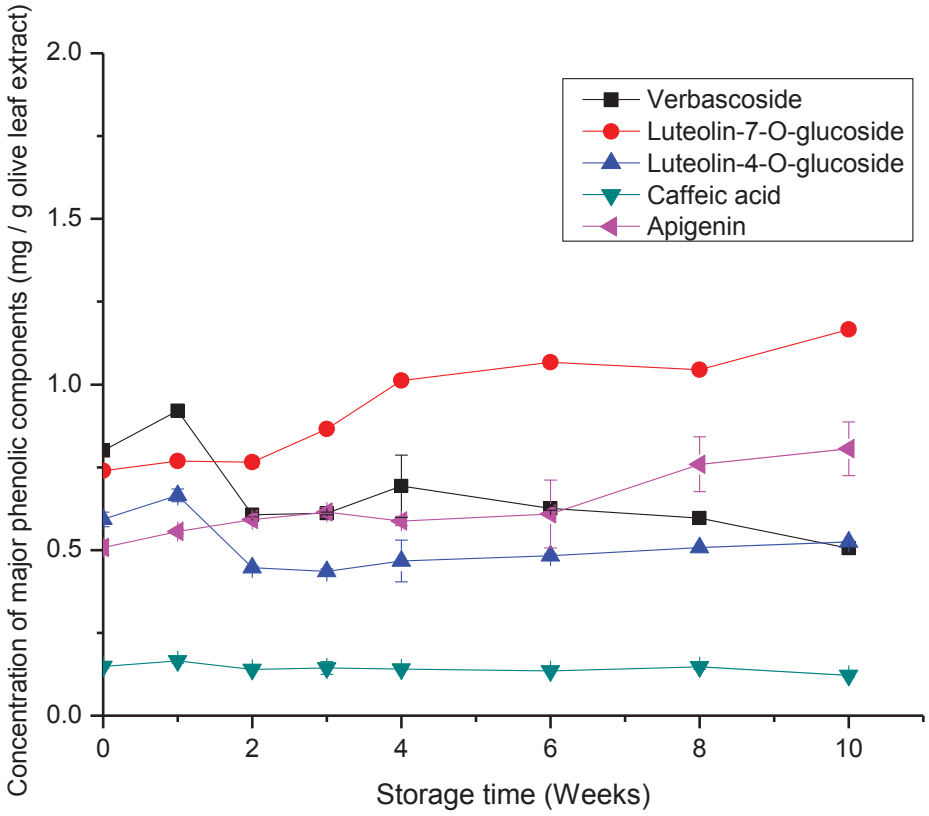



Figure 4.26.b: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at 4°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

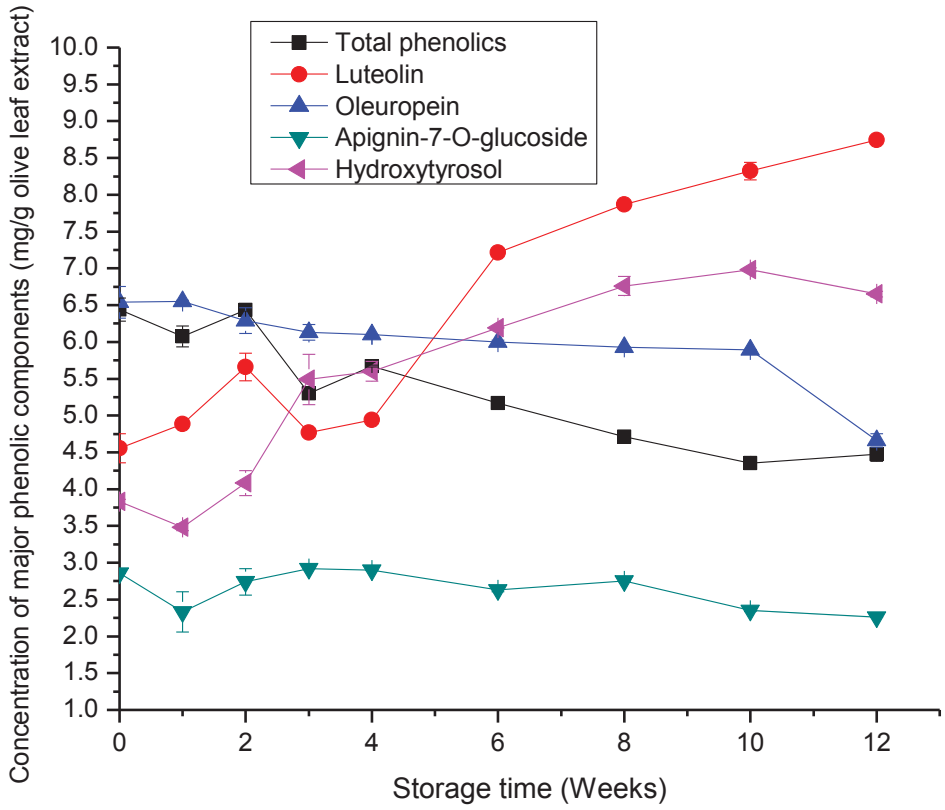



Figure 4.27.a: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at 20°C, All Experiments Were Carried Out in Duplicates and Data Points Are Mean ±SEM (N=2)

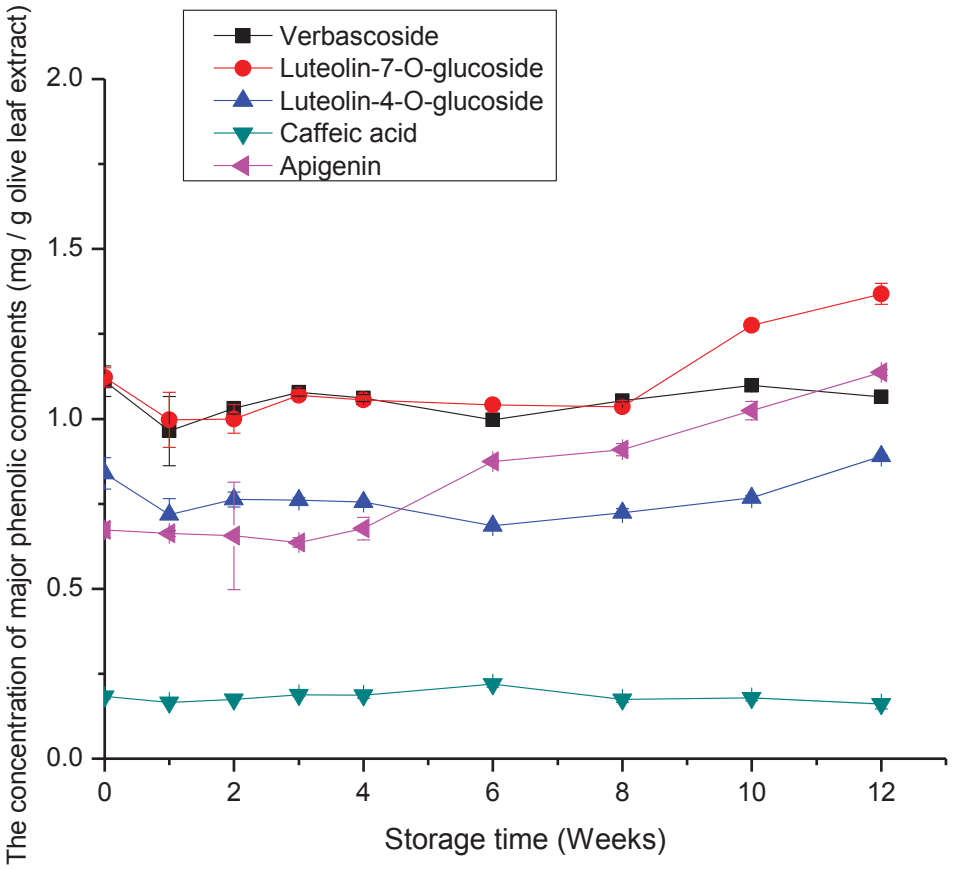



Figure 4.27.b: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at 20°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

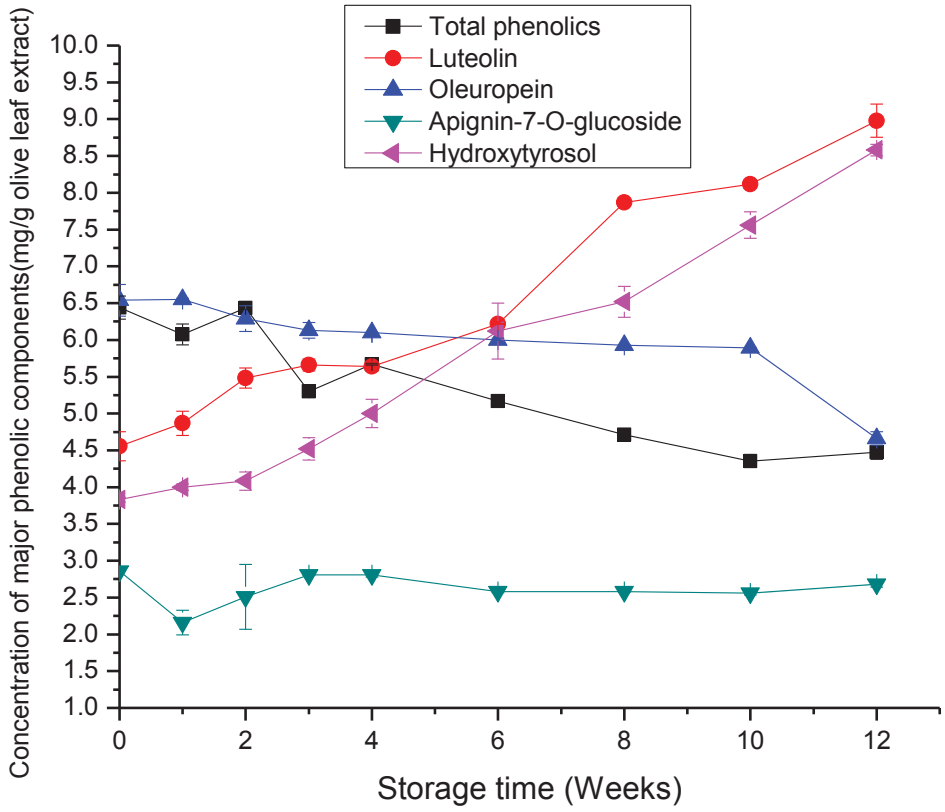



Figure 4. 28.a: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at 30°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

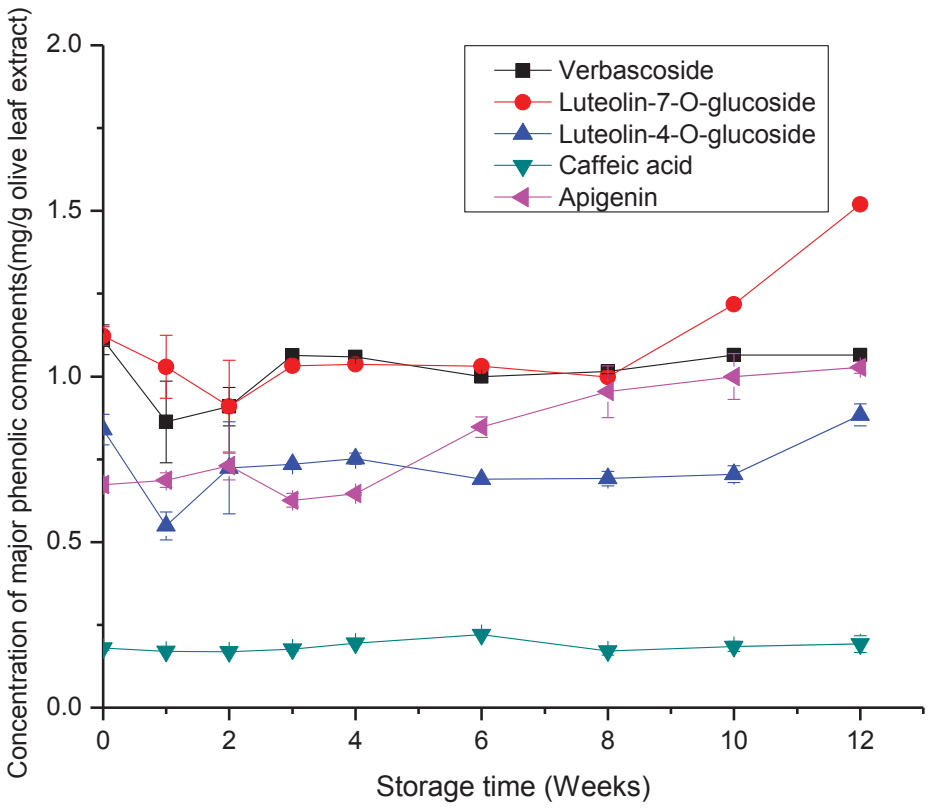



Figure 4.28.b: The Concentration Change of Major Phenolic Components of 80% Ethanol Based Olive Leaf Extract When Stored at 30°C, All Experiments Were Carried out in Duplicates and Data Points Are Mean ±SEM (N=2)

The published studies on the stability of olive leaf extracts are rather limited, and the pathway of degradation of phenolic compounds, in particular, oleuropein has been investigated exclusively for olive fruit. Two routes of hydrolysis were suggested by Ryan et al. (2002) for the degradation of oleuropein during maturation of olives. Firstly, cleavage by specific esterase enzymes gives rise to either elenolic acid glucoside or demethyloleuropein, which are both found in ripe olives. Alternatively, it has been assumed that crushing and malaxation of olive fruits during oil production activates the endogenous β-glucosidase, which produce the aglycone. Demethyloleuropein derived from esterase activity may also act as a substrate for β-glucosidase (Ryan et al., 2002). The oleuropein aglycone obtained can be further convert to hydroxytyrosol by subsequent esterolysis.

The glucosidases are a widespread group of enzymes with significant biochemical, biomedical, and industrial importance that catalyze the hydrolysis of glycosidic bonds in oligo- and polysaccharides, and β-glucosidase from olive fruit tissues is a key enzyme in fruit ripening (Briante et al., 2001).

Oleuropein can be hydrolysed to hydroxytrosol, elenoic acid, and oleuropein aglycone. Hydrolysis can be achieved either chemically or enzymatically (Mourtzinos et al., 2007). Chemical hydrolysis of oleuropein can be carried out by using HCl at 100°C on olive leaves or olive leaf extracts, in such a way, high concentrations of hydroxytrosol can be obtained (De Leonardis et al., 2008; Jemai et al.,2008). Enzymatically hydrolysis of oleuropein can be conducted by using endogenous β-glucosidase present in olive juice or by a commercial β-glucosidase on olive leaf extract (Briante et al., 2001). The temperature of experimental condition was 37°C and the pH of mixture was 7. The biotransformation of olive leaf extract by β-glucoside showed a small amount of hydroxytrosol and high concentration of oleuropein aglycone (Jemai et al., 2008).

Two chemical reactions may be involved in degradation of phenolic compounds during storage in this study, which are hydrolysis and oxidation. The enzymes involved in degradation are β-glucosidase and polyphenol oxidise (PPO). Oxygen is required for both enzymatic and non-enzymatic oxidation as it is a co-substrate for PPO (Obied et al., 2005).

A patent of olive leaf extraction suggested olive enzyme may be inactivated by drying at 100°C for 1-2 hours or in a low temperature (50-60°C) for more than 24 hours, or placing dry materials in boiling water or alcohol for 30 minutes, freeze drying or pre-treatment of the dry materials with acid at low temperature (<20°C) (Pinnell et al, 2003). In this study, fresh olive leaves were dried at 30°C for 48 hours, then ground and extracted with 80% ethanol at 40°C for 24 hours, however, β-glucosidase in leaf extracts may be activated during extraction and still active under all storage conditions, which may explain the decrease of some of the phenolic compounds such as oleuropein and apigenin-7-glucoside. The pH of concentrated olive leaf extract was around 6.0, and the enzymatic reaction may be favoured under these weak acidic conditions, where the enzymes and the substrates are in close contact. It was reported in the patent (Pinnell et al,2003) that β-glucosidase starts to be active from 37°C to 41°C, which may explain the greatest extent of phenolic degradation and hence largest increase in metabolite concentration was observed in the samples stored at 30°C (Pinnell et al.,2003).

Obied et al. (2005) suggested that the presence of oxygen seems to be essential in the degradation of verbascoside, and verbascoside has good stability toward acid hydrolysis. As oxygen was eliminated as much as possible from the samples, this may explain why verbascoside remained relatively stable in this study (Obied et al., 2005).

When comparing the stability of 80% and 60% ethanol based extracts, under the same storage conditions, the only difference between the two samples was the starting concentration of total phenolics, resulting in different substrate concentrations during enzymatic hydrolysis degradation. The oleuropein in the original 80% and 60% olive leaf extracts was 6.54 mg/g and 3.94 mg /g respectively, and this may explain partly the difference in the extent of hydrolysis degradation observed in two extracts.

### 4.9.6 Correlation between Total Phenolic Content, Individual Phenolic Compounds and Antioxidant Capacity

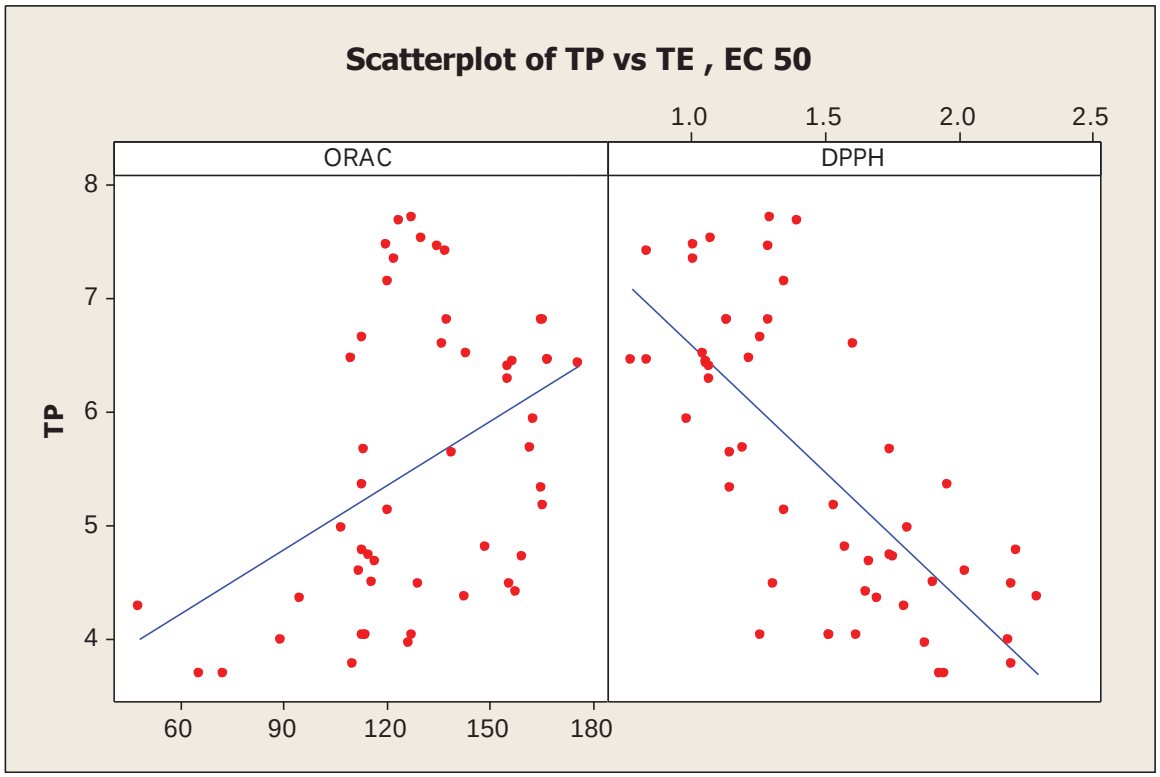



Figure 4. 29: The Correlation between Total Phenolics and Antioxidant Capacity, All Data Points Are Mean Values of Triplicates. (N=3)

Figure 4.29 was obtained by plotting mean value of total phenolics of olive leaf samples stored at all four temperatures against TE values (ORAC) and EC values (DPPH) at corresponding temperatures. The results indicate degree of correlation between total phenolic and antioxidant capacity measured by DPPH and ORAC assays. The regression analysis shows that there was no correlation ($R^2$ =0.17) and between antioxidant capacities and total phenolic content with ORAC assay. A negative correlation ($R^2$ =0.52) was found between antioxidant capacities and total phenolic content with DPPH assays. Olive leaf extracts with higher total phenolic content are more effective on scavenging free radicals.

Generally, the antioxidant capacity expressed as EC50 (DPPH assay) of leaf extracts and the total phenol content showed a good correlation. However, a poor linear correlation was found between total phenolics and antioxidant capacity in this project, which may be due to phenolic profile in extracs. A phenolic profile is considered as important in relation to total antioxidant capacity of olive leaf extract (Kiritsakis et al., 2010). DPPH radical is a very weak oxidising agent, and is not sufficiently strong to oxidise compounds with more isolated phenolic group (e.g. courmaric acid), which might still be oxidized by strong oxidizing agent such as Folin-Ciocalteau reagent. This is likely another cause of poor correlation between total phenolics and antioxidant capacity (EC50) in this project.

Comparing the TE value and EC50 of leaf extracts obtained using 80% ethanol with 60% ethanol before storage, the antioxidant capacity of 80% ethanol based leaf extracts was higher than 60% ethanol based leaf extract , which corresponded to higher levels of total phenolics in the 80% ethanol based leaf extracts.

Moure et al. (2001) reported that the antioxidant capacity depends on the extract concentration. As a general trend, increased antioxidant capacity was found with increasing extract concentration, but the concentration leading to maximum antioxidant activity is closely dependent on the antioxidant capacity assays used (Moure, et al., 2001)

## 4.10 Evaluation of Microbiological Safety of Olive Leaf Extract

The results of standard plate count at the beginning, in the middle and at the end of storage show zero CFU/ml for all concentrated olive leaf extracts samples, which indicates there were no microbial hazards existing in olive leaf extracts from this study.

The bactericidal effect of oleuropein and its hydrolysis products was observed against a broad spectrum of other Gram-positive and Gram negative bacteria, but no effect was

observed against yeast (Soler-Rivas, 2000). It may imply that microorganisms may not easily grow in the olive leaf extracts, but yeast may be an issue in the storage of olive leaf extracts.

## 4.11 Comparison of Oleuropein Concentrations between Olive Leaf Extracts With Commercial Products

The oleuropein concentrations of two commercial olive leaf extracts products were measured using the method in Section 3.9. Commercial olive leaf extract products in New Zealand market are either in liquid form or in capsule form. Two commercial products were tested in order to compare oleuropein level with that of concentrated olive leaf extracts obtained in this study. As showen in the Table 4.11, oleuropein determined in the 'Oliviral' olive leaf extract (manufacturer: Nutrilife) contains 47.7 mg Oleuropein per capsule, which is similar with the amount claimed on product label. Oleuroepin determined in 5ml Comvita olive leaf extracts is 24.03mg, which is also similar to the value claimed on their product label. The amount of oleuropein in 5ml concentrated olive leaf in this study is 25.86mg. The oleuropein in the capsule form products are generally higher than that in liquid form according to the information provided by manufactures. The ranges of oleuropein in liquid form products on the market are from 16mg to 32 mg per 5ml extract, which indicates olive leaf extract obtained in this study contains good level of oleuropein.

Table 4. 11: Oleuropein Concentrations in Three Olive Leaf Extracts

| **Olive leaf extract** | **Oliviral** | **Comvita** | **80% Ethanol based concentrated extracts** |
|---|---|---|---|
| Oleuropein concentrations claimed on the label | 48.67mg/g | 22 mg/ 5ml | n/a |
| Oleuropein concentrations measured in the study | 47.4±0.35 mg/g | 24.03±0.01mg/5ml | 25.86±0.05 /5ml |

# Chapter Five: Conclusions and Recommendations

Olive leaves are a good and cheap source of phenolic compounds. The recovery of phenolic compounds from olive leaves could be achieved by solvent extraction. The solvent should be low toxicity, economical and easily recycled by evaporation. Industrially, the feasibility of the extraction process involves the search for the optimum extraction conditions to maximize the efficiency of the process. In this study, aqueous ethanol, boiling water and ultrasonic solvent extraction were investigated to identify optimum extraction conditions. It was found that higher total phenolic content and oleuropein concentration were obtained by increasing temperature and solvent: solid ratio. The results show that good recovery of phenolic compounds can be achieved using 80% ethanol (v/v) at a temperature of 40°C and solvent: solid ratio of 30. The ethanol concentration significantly influences oleuropein concentration ($P \leq 0.05$). More oleuropein was recovered using increasing percentage of ethanol in the solvent mixture. Boiling water showed a poor recovery of phenolic compounds with extraction efficiency of 40%, which was only half of recovery obtained by 80% ethanol (v/v). Ultrasound can dramatically accelerate extraction rate and reduce the extraction time, however it may also increase the cost.

There were six main phenolic compounds identified in the olive leaf extracts, namely oleuropein, hydroxytyrosol, luteolin-7-glucoside, luteolin-4-glucoside, apigenin-7-O-glucoside, verbascoside. Oleuropein was predominant phenolic compound in the olive leaf extract. Apart from oleuroepin, high amounts of luteolin-7-O-glucose, luteolin-4-O-glucoside and apigenin-7-O-glucose were also present in olive leaf extracts obtained in this study, which may suggest that the leaves used in the study were mature leaves. It was found that much better recovery of phenolic compounds was achieved using combination of alcohol and water than using water alone. There was significant difference ($P \leq 0.05$) in oleuropein recovered between 80% ethanol (v/v) and boiling water, boiling water only recovered 36% oleuropein of that recovered by alcoholic solvent. Cultivar and collection time are important factors and have impact on the phenolic compounds extracted, though the results of the effect of cultivar in this study are inconclusive. It was found in this study that drying significantly ($P \leq 0.05$) improves the oleuropein concentration in the extract. Drying fresh leaves at 30°C is a good method for leaf pre-treatment prior to extraction.

The olive leaf extract was to be effective in the function of scavenging free radicals according to the results determined by two antioxidant activity assays in this study.

Temperature significantly affected ($P \leq 0.05$) the degradation of phenolic compounds during storage, consequently influenced antioxidant activity of olive leaf extract. It was found that greater extent of degradation of oleuroepin and a decrease in antioxidant activity occurred with increasing temperatures.

Based on the finding in this study, the recommendations are given as follows:

- Sampling

Oleuropein were markedly affected by the colour and age factor, which was higher in the leaves with an intense green colour including mainly those at developing stage. The green-yellowish leaves and noticeably the yellow ones had lower contents of oleuropein. Oleuropein in olive leaves is gradually degraded with their progressive aging, due most probably to endogenous β-glycoside enzyme. It is recommended that green and relatively young olive leaves are required in order to obtain extracts with higher level of oleuropein.

- Post-harvest treatment

Drying leaves are highly recommended before extraction in order to decrease water activity and reduce enzymatic destruction of some polyphenols but also increases the synthesis of Oleuropein. The leaves should be dried as soon as possible to eliminate hydrolysis of oleuropein. Drying can be carried out using conventional or ventilated ovens with microwave assisted heating. Obviously avoidance of high temperature and long air exposure times can effectively avoid changes due to enzymatic activities. Dried leaves are preferably kept in sealed containers (glass) and stored in a cool and dark place.

- Extraction procedure

Agitation is necessary during extraction process, and it can be achieved with assistant of ultrasound or mechanical shaker. In this study, the possible oxidation due to PPO activity during extraction was not monitored. The most effective way to control PPO activity is the use of antioxidants or reducing agents and elimination of oxygen.

- Storage conditions

The olive leaf extract is recommended to be stored under conditions in which oxygen and and light and a temperature of -20°C is recommended for storage. Three month storage at room temperature for liquid olive leaf extracts can lead to considerable loss of phenolic

compounds, and thus prolong storage should be avoided for liquid olive leaf extracts. It is recommended that encapsulation with aids of hydrocolloids in capsule may prolong shelf life of product.

Olive leaf is a promising source for exploitation in terms of its bioactive content. These results in this study could mean the first step for the implementation of the process on a large scale, being an adequate starting point for further studies regarding the optimization of the continuous process, of major interest from an industrial point of view.

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# Appendices

## Appendix 3. 1: Determination of DPPH$^{\cdot}$ Radical Scavenging Activity of Olive Leaf Extract

Reagent

DPPH ($1.5X10^{-4}M$): completely dissolve 0.0597 gram of 2, 2-diphenyl-1-picryhydrazyl radical (Sigma) in 1L of absolute methanol (Thermo Fisher), store DPPH radical solution at $4^0C$ in the dark

Procedure

- Weight 0.5g concentrated olive leaf extract, and dissolve in 50ml 60% or 80% ethanol, making final concentration to 10mg/ml
- A range of dilutions of olive leaf extract samples (150µg/mL to 2500µg/ml) are prepared by pipetting the following volumes of 10mg/ml original leaf extract and making up to 4 mL with absolute methanol in falcon tubes: 60 µl to 1000µl.
- DPPH radical solution (10 mL) was added into each of diluted olive leaf extract samples. The capped tubes were incubated in the dark at 20 ±0.5°C and absorbance was measured at 520nm in triplicate using a Shimadzu UV-1700 spectrophotometer after 40 minutes.
- For a control sample, 4 ml methanol was used and then followed same procedure as above.

Calculation of Result

$$\%\text{Radical scavenging activity} = (A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}} \times 100\%$$

The percentage of DPPH radical scavenging activity was plotted against the plant extract concentration (µg/ mL) to determine the amount of extract necessary to decrease the DPPH radical concentration by 50% (called EC50). An example is given in below Table 1 and Figure 1.

Table 1: The DPPH scavenging activity of olive leaf extracts at different concentrations

| Sample | Concentration (µg/ml) | ABS at 520 | %Radical scavenging activity |
|---|---|---|---|
| | Control | 1.215 | 0 |
| W4-60%-4C | **150** | **0.986** | **18.85** |
| | 450 | 0.852 | 29.88 |
| | 550 | 0.755 | 37.86 |
| | 750 | 0.45 | 62.96 |
| | 1250 | 0.196 | 83.87 |

The concentration of olive leaf extracts were calculated from a ranage of dilution of original olive leaf sample, which has the concentration of 10mg/ml.

For example: 600µl olive leaf extracts diluted with 3.4 ml methanol in test tube, which made the total volume of diluted sample up to 4ml , and thus the concentration of diluted olive leaf extracts was 0.15mg/ml.

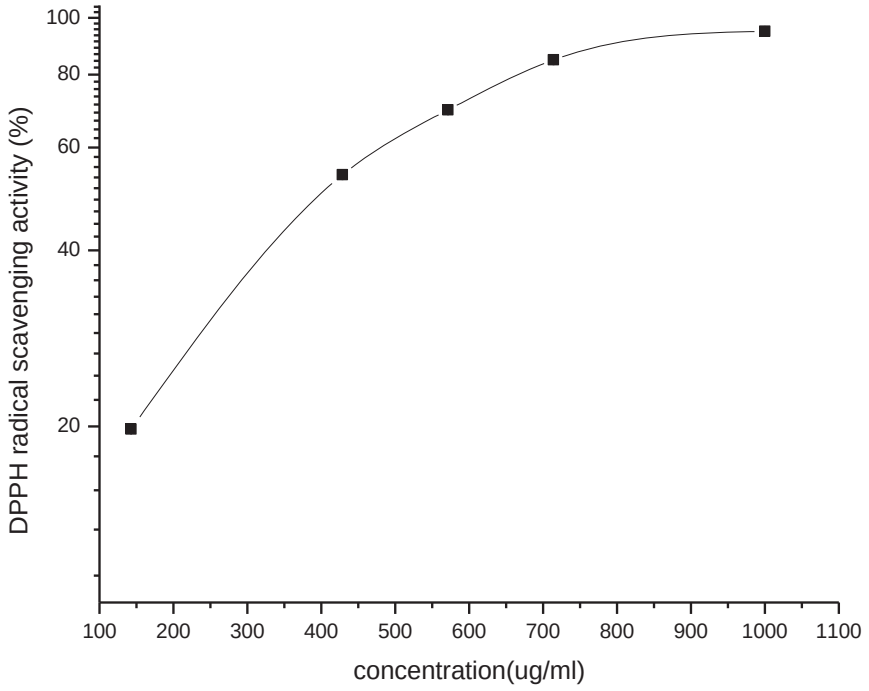



Figure 1: DPPH radical scavenging activity of the ethanolic extracts of dried olive leaves

**% DPPH Radical scavenging activity = (1.215-0.986)/1.215x100%=18.85%**

Plot % DPPH radical scavegening activity agaist all five concentration, then approximately 400µg/ml of olive leaf extract was required to achive 50% DPPH radical scavenging activity, thus EC 50= 400µg/ml

## Appendix 3. 2: Determination of Oxygen Radical Absorbance Capacity (ORAC) of Olive Leaf Extract

Reagent

Phosphate buffer (75mM, pH 7.4): completely dissolve 2.338 g of monosodium phosphate, monohydrate ($NaH_2PO_4.H_2O$) and 8.242 gram of disodium phosphate ($Na_2HPO_4$) in 1L of distilled water, store buffer solution at room temperature.

AAPH (153 mM): 0.414 g of 2, 2'-Azobis (2-amidinopropane) dihydrochloride (Cayman chemicals, USA) was completely dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 153 mM and was kept in an ice bath. The unused AAPH solution was discarded within 8 hours.

Sodium Fluorescein solution: A fluorescein stock solution ($4 \times 10^{-3}$ mM) was made by dissolving 0.075 g fluorescent salt ($C_{20}H_{10}Na_2O_5$) (Sigma-Aldrich, NZ) in 50ml phosphate buffer (pH 7.4) and stored at 4°C in the dark. Immediately prior to use, the stock solution was diluted 1:1000 with 75 mM phosphate buffer (ph 7.4). The diluted sodium fluorescein solution was made fresh daily.

Standard Trolox solution (0.02M) : 0.250 g of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Sigma-Aldrich, NZ) was dissolved in 50 mL of 75 mM phosphate buffer (pH 7.4) to give a 0.02 M stock solution. The stock solution was diluted with the same phosphate buffer to 6.25 $\mu$M, 12.5 $\mu$M, 25w $\mu$M, 50 $\mu$M and 100$\mu$M working solutions.

Equipments:

FLUOstar Optima automated micro plate reader (BMG Labtech, Germany) with 96-well plates with fluorescence filters for an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Procedure

- Weight 0.5g concentrated olive leaf extract, and dissolve in 50ml 60% or 80% ethanol, making final concentration to 10mg/ml

- To each well 25 µl of olive leaf samples or 75 mM phosphate buffer (pH 7.4) (blank) or standard (Trolox) were added to 150 µl fluorescein solution.
- The plate was then allowed to equilibrate by incubating for a minimum of 10 minutes in the incubator at 37°C.
- Reactions were initiated by the addition of 25 µl of 153mM AAPH solution for a final reaction volume of 200 µl. The fluorescence was then monitored kinetically with data taken every minute for a total of 70 minutes.

Calculation of Result

- Plot fluorescence intensity of blank or sample or standard against time to obtain a fluorescence(FL) decay curve

- Calculate the area under FL curve (AUC) and the Net AUC of the standards ($AUC_{standard}$ - $AUC_{blank}$) and olive leaf extract samples ($AUC_{sample}$ - $AUC_{blank}$) using Equations below

$$AUC = 0.5 + f_1/f_0 + \ldots f_i/f_0 \ldots + f_{59}/f_0 + f_{70}/f_0$$

Where $f_0$, $f_i$, $f_{70}$ are the fluorescent readings at 0 minutes, i minutes and 70 minutes

- Plot Net AUC of Trolox at five concentration levels against the concentration of Trolox , and obtain a Trolox calibration curve and regression Equation

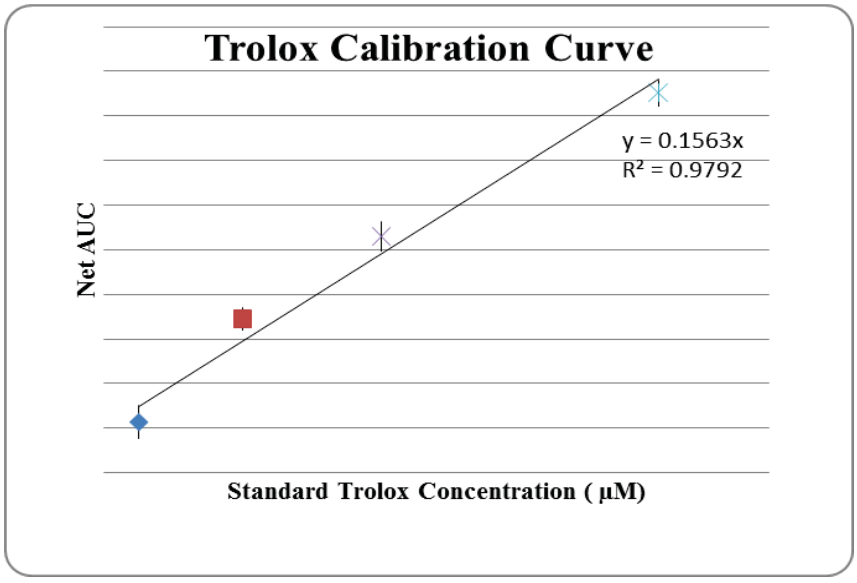



Figure 2:Trolox Calibartion Curve for Determination of Oxygen Radical Absorbance Capacity (ORAC) of Olive Leaf Extract

Where Y is Net AUC of Standard Trolox and X is the concentration of Trolox

- ORAC value of olive leaf extract was calculated from Equation below and expressed as micromoles of Trolox equivalents (TE) per gram of extract (µmol of TE $g^{-1}$).

$$\text{Trolox equivalents (µmol of TE/g olive leaf extract)} = \frac{(\text{Net AUC of Leaf extract sample} - \text{Intercept}) \times \text{Dilution factor}}{\text{Slope} \times \text{Wt of olive leaf extract}}$$

## Appendix 4. 1: ANOVA Table For Total Phenolics

General Linear Model: total phenolics versus con, temp, ratio

| Factor Type | | Levels | Values |
|---|---|---|---|
| Concentration | fixed | 3 | 60, 70, 80 |
| Temperature | fixed | 2 | 20, 40 |
| Solvent-to-solid ratio | fixed | 2 | 20, 30 |

Analysis of Variance for total phenolics, using Adjusted SS for Tests

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Concentration | 2 | 4.866 | 4.866 | 2.433 | 2.22 | 0.130 |
| Temperature | 1 | 191.890 | 191.890 | 191.890 | 175.42 | 0.000 |
| Solvent-to-solid ratio | 1 | 53.005 | 53.005 | 53.005 | 48.45 | 0.000 |
| Con*temp | 2 | 0.107 | 0.107 | 0.054 | 0.05 | 0.952 |
| Con*ratio | 2 | 1.683 | 1.683 | 0.842 | 0.77 | 0.474 |
| Temp*ratio | 1 | 0.193 | 0.193 | 0.193 | 0.18 | 0.678 |
| Con*temp*ratio | 2 | 1.521 | 1.521 | 0.761 | 0.70 | 0.509 |
| Error | 24 | 26.254 | 26.254 | 1.094 | | |
| Total | 35 | 279.519 | | | | |

S = 1.04590 R-Sq = 90.61% R-Sq (adj) = 86.30%

Regression Analysis: total phenolics versus Temperature, Solvent-to-solid ratio

The regression Equation is total phenolics = 9.75 + 0.231 Temp + 0.243 Ratio

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 9.754 | 1.010 | 9.66 | 0.000 |
| Temp | 0.23087 | 0.01707 | 13.52 | 0.000 |
| Ratio | 0.24268 | 0.03414 | 7.11 | 0.000 |

S = 1.02431 R-Sq = 87.6% R-Sq(adj) = 86.9%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 244.90 | 122.45 | 116.70 | 0.000 |
| Residual Error | 33 | 34.62 | 1.05 | | |
| Total | 35 | 279.52 | | | |

**One-way ANOVA: total phenolics versus Temp**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|

Temp 1 191.89 191.89 74.45 0.000
Error 34 87.63 2.58
Total 35 279.52

S = 1.605 R-Sq = 68.65% R-Sq(adj) = 67.73%

Individual 95% CIs For Mean Based on Pooled StDev

Level N Mean StDev -------+---------+---------+---------+--
20 18 20.438 1.669 (----*----)
40 18 25.056 1.540 (----*---)
-------+---------+---------+---------+--
20.8 22.4 24.0 25.6

Pooled StDev = 1.605

**One-way ANOVA: total phenolics versus Ratio**

Source DF SS MS F P
Ratio 1 53.00 53.00 7.96 0.008
Error 34 226.51 6.66
Total 35 279.52

S = 2.581 R-Sq = 18.96% R-Sq(adj) = 16.58%

Individual 95% CIs For Mean Based on Pooled StDev

Level N Mean StDev -----+---------+---------+---------+----
20 18 21.533 2.634 (--------*-------)
30 18 23.960 2.528 (--------*-------)
-----+---------+---------+---------+----
21.0 22.5 24.0 25.5

Pooled StDev = 2.581

**One-way ANOVA: total phenolics versus Concentration**

Source DF SS MS F P
Concentraiton 2 4.87 2.43 0.29 0.748
Error 33 274.65 8.32
Total 35 279.52

S = 2.885 R-Sq = 1.74% R-Sq(adj) = 0.00%

Individual 95% CIs For Mean Based on Pooled StDev

```
Level   N    Mean  StDev  ---------+---------+---------+---------+
60      12  22.893  2.788        (-------------*-------------)
70      12  22.242  2.816  (-------------*-------------)
80      12  23.106  3.044         (--------------*-------------)
                    ---------+---------+---------+---------+
                          21.6      22.8      24.0      25.2
```

Pooled StDev = 2.885

## Appendix 4. 2: ANOVA Table for Oleuropein Concentration

General Linear Model: Oleuropein versus Blocks, Temp, Ratio, Con

| Factor | Type | Levels | Values |
|---|---|---|---|
| Blocks | fixed | 3 | 1, 2, 3 |
| Temperature | fixed | 2 | 20, 40 |
| Solvent-to-solid Ratio | fixed | 2 | 20, 30 |
| Concentration | fixed | 3 | 60, 70, 80 |

Analysis of Variance for oleuropein, using Adjusted SS for Tests

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Temperature | 1 | 1682402397 | 1682402397 | 1682402397 | 2535.35 | 0.000 |
| Solvent-to-solid ratio | 1 | 301002312 | 301002312 | 301002312 | 453.61 | 0.000 |
| Concentration | 2 | 153038131 | 153038131 | 76519065 | 115.31 | 0.000 |
| Temp*Ratio | 1 | 21667974 | 21667974 | 21667974 | 32.65 | 0.000 |
| Temp*Con | 2 | 8050802 | 8050802 | 4025401 | 6.07 | 0.008 |
| Ratio*Con | 2 | 1493948 | 1493948 | 746974 | 1.13 | 0.339 |
| Temp*Ratio*Con | 2 | 12115842 | 12115842 | 6057921 | 9.13 | 0.001 |
| Error | 22 | 14598716 | 14598716 | 663578 | | |
| Total | 35 | 2195608257 | | | | |

S = 814.603 R-Sq = 99.34% R-Sq(adj) = 98.94%

Regression Analysis: Oleuropein versus Temperature, Solvent-to-solid ratio, Concentration

The regression Equation is
Oleuropein = - 23058 + 684 Temp + 578 Ratio + 251 Con

| Predictor | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | -23058 | 2388 | -9.66 | 0.000 |
| Temperature | 683.62 | 22.92 | 29.82 | 0.000 |
| Solvent-to-solid Ratio | 578.31 | 45.85 | 12.61 | 0.000 |
| Concentration | 251.39 | 28.08 | 8.95 | 0.000 |

S = 1375.40 R-Sq = 97.2% R-Sq(adj) = 97.0%

Analysis of Variance

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 3 | 2135072703 | 711690901 | 376.21 | 0.000 |

Residual Error 32 60535554 1891736
Total 35 2195608257

**One-way ANOVA: olea versus Temp**

Source DF SS MS F P
Temp 1 1682402397 1682402397 111.46 0.000
Error 34 513205860 15094290
Total 35 2195608257

S = 3885 R-Sq = 76.63% R-Sq(adj) = 75.94%

Individual 95% CIs For Mean Based on Pooled StDev

Level N Mean StDev --------+---------+---------+---------+-
20 18 22669 4591 (--*---)
40 18 36342 3018 (---*--)
--------+---------+---------+---------+-
25000 30000 35000 40000

Pooled StDev = 3885

**One-way ANOVA: olea versus Ratio**

Source DF SS MS F P
Ratio 1 301002312 301002312 5.40 0.026
Error 34 1894605945 55723704
Total 35 2195608257

S = 7465 R-Sq = 13.71% R-Sq(adj) = 11.17%

Individual 95% CIs For Mean Based on Pooled StDev

Level N Mean StDev ----+---------+---------+---------+-----
20 18 26614 8202 (---------*---------)
30 18 32397 6646 (----------*---------)
----+---------+---------+---------+-----
24500 28000 31500 35000

Pooled StDev = 7465

**One-way ANOVA: olea versus Con**

Source DF SS MS F P
Con 2 153038131 76519065 1.24 0.304

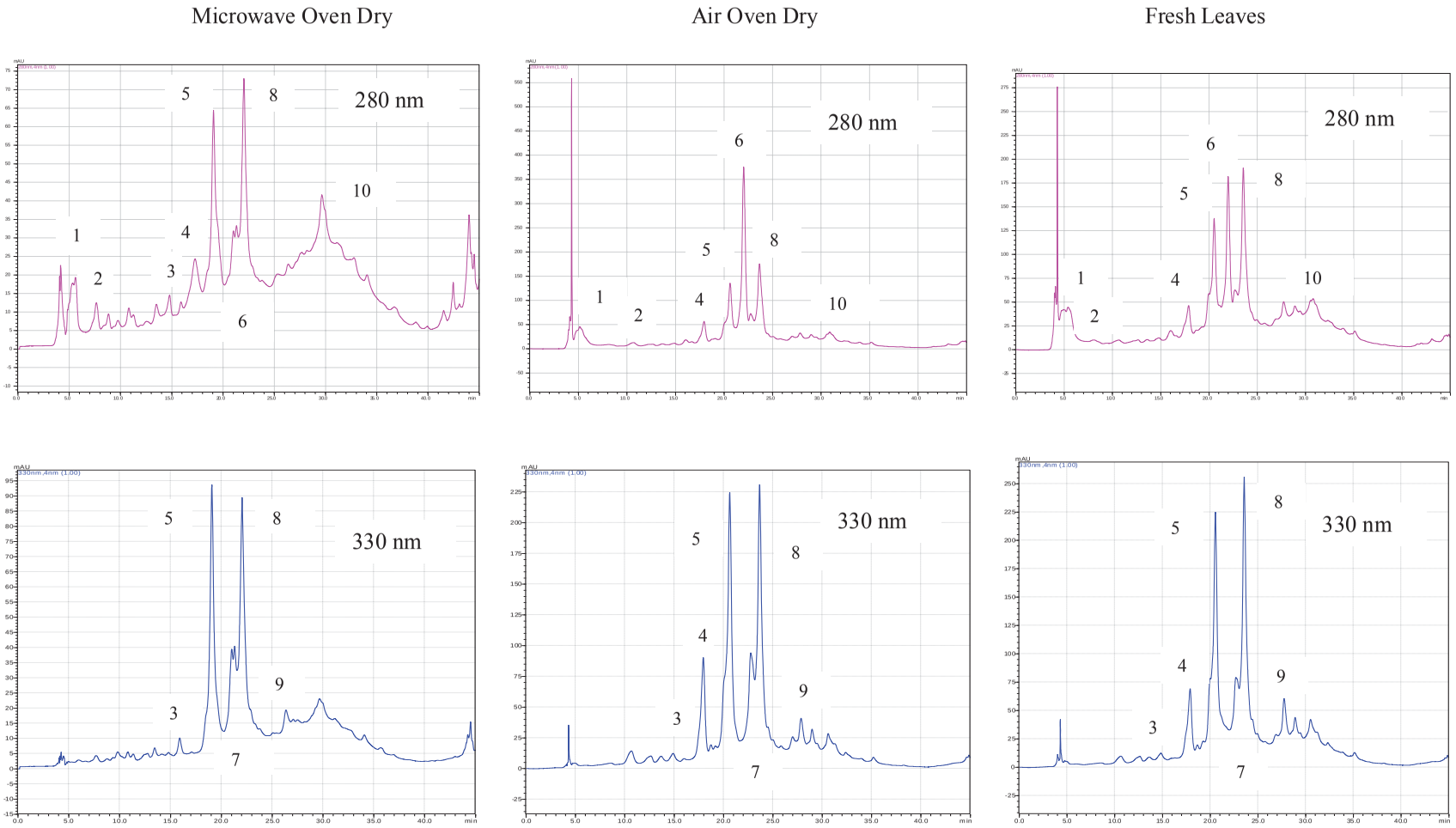
```
Error   33  2042570126  61896064
Total   35  2195608257

S = 7867   R-Sq = 6.97%   R-Sq(adj) = 1.33%


                    Individual 95% CIs For Mean Based on
                    Pooled StDev
Level   N   Mean  StDev  ----+---------+---------+---------+-----
60     12  27130   8555  (-----------*----------)
70     12  29230   7530        (----------*-----------)
80     12  32157   7470              (----------*-----------)
                    ----+---------+---------+---------+-----
                    24000     28000     32000     36000

Pooled StDev = 7867
```

## Appendix 4. 3: Equation for calibration curve, correlation coefficient and concentration range for linearity for phenolic compounds

| Phenolic compounds | Equation | Correlation coefficient | Linearity (mg/ml) |
|---|---|---|---|
| Oleuropein | $Y = 1.21182*10^7X$ | 0.99 | 0.5-2 |
| Luteolin-7-O-glucoside | $Y = 5.8358*10^7X$ | 0.99 | 0.0125-0.05 |
| Luteolin-4-O-glucoside | $Y = 6.41727*10^7X$ | 0.99 | 0.0125-0.05 |
| Rutin | $Y = 4.27802*10^7X$ | 0.99 | 0.0125-0.05 |
| Apignin-7-o-glucoside | $Y = 7.85972*10^7X$ | 0.99 | 0.0125-0.05 |
| Verbascoside | $Y = 5.3081*10^7X$ | 0.99 | 0.0125-0.05 |

Y: peak area at 245nm, 280nm, 320nm, 330nm or 340nm; X –concentration of phenolic compounds; R square-correlation efficiency

## Appendix 4. 4 Chromatogram of Dried Olive Leaves Vs. Fresh Leaves




HPLC profile of fresh olive leaf extract (Frantoio cultivar) vs. olive leaf extracts using two drying methods: (1) hydroxytyrosol; (2)tyrosol ;(3) verbascoside; (4) rutin ;(5) luteolin 7-O-glucoside; (6) oleuropein; (7) luteolin-4-O-glucoside; (8) apigenin 7-O-glucoside; (9) luteolin; (10 ) apigenin; Detection was at 280nm and 330nm

Fresh olive leaf (Frantoio cultivar)

Collected in November

Microwave oven dried olive leaf (Frantoio cultivar)

Drying condition:

2 minutes at maximum power of 1000W

Air oven dried olive leaf (Frantoio cultivar)

Drying condition:

48 hours at 30 ± 0.5°C in an incubator

Pictures of different olive leaf sample

## Appendix 4. 5: Mass Balance calculation

### 1. The percentage of Wt loss during concentration

Wt of original olive leaf extract before concentration: 50.015g

The density of original extract: **0.8704g/ml**

The volume of original olive leaf extract before concentration: 57.46ml

Wt of concentrated leaf extract: 5.260g

The density of aqueous extract: **1.0117 g/ml**

The volume of concentrated olive leaf extract after concentration: 5.10ml (10 fold)

Wt of ethanol evaporated: 42.914g

Wt of extract before concentration

= Wt of extract after concentration + Wt of ethanol evaporated+ weight loss

Weight loss= 50.015-5.260-42.914=1.841g

The percentage of Wt loss during concentration: 1.841/ 50.015*100=3.68%

### 2. Mass balance of total phenolic content and oleuropein

The total phenolics in original extract (mg caffeic acid/ml): 0.64

$$\frac{0.64\text{mg}}{ml} \times \frac{ml}{0.8704g} \times 50.015g = 36.78g$$

The total phenolics in concentrated extract (mg caffeic acid/ml): 6.85

$$\frac{6.85\text{mg}}{ml} \times \frac{ml}{1.0117g} \times 5.260g = 35.61g$$

The loss of total phenolic content: (36.78-35.61)/36.78×100=**3.18%**

The oleuropein in original extract: 0.48 mg/ml

$$\frac{0.48\text{mg}}{ml} \times \frac{ml}{0.8704g} \times 50.015g = 27.582g$$

The oleuropein in concentrated extract: 5.07mg/ml

$$\frac{5.07\ \text{mg}}{ml} \times \frac{ml}{1.0117g} \times 5.260g = 26.360g$$

The loss of oleuropein: (27.582-26.360)/27.582×100=**4.43%**

## Appendix 4. 6 :Two Sample t-Test Results For 60% Ethanol Based Olive Leaf Extracts

1. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at -20° C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 4.0190 | 0.0300 | 0.017 |
| 2 | 3 | 4.270 | 0.160 | 0.092 |

Difference = mu (1) - mu (2)
Estimate for difference: -0.2510
95% CI for difference: (-0.6032, 0.1012)
T-Test of difference = 0 (vs not =): T-Value = -2.67 P-Value = **0.056** DF = 4
Both use Pooled StDev = 0.1151

2. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at 4 °C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 4.0190 | 0.0300 | 0.017 |
| 2 | 3 | 3.6860 | 0.0210 | 0.012 |

Difference = mu (1) - mu (2)
Estimate for difference: 0.3330
95% CI for difference: (0.2538, 0.4122)
T-Test of difference = 0 (vs not =): T-Value = 15.75 P-Value = **0.000** DF = 4
Both use Pooled StDev = 0.0259

3. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at 20 ° C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 4.800 | 0.370 | 0.21 |
| 2 | 3 | 3.340 | 0.260 | 0.15 |

Difference = mu (1) - mu (2)
Estimate for difference: 1.460
95% CI for difference: (0.482, 2.438)
T-Test of difference = 0 (vs not =): T-Value = 5.59 P-Value = **0.005** DF = 4
Both use Pooled StDev = 0.3198

4. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at 30 °C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 4.800 | 0.370 | 0.21 |

2 3 2.973 0.260 0.15

Difference = mu (1) - mu (2)
Estimate for difference: 1.827
95% CI for difference: (0.849, 2.805)
T-Test of difference = 0 (vs not =): T-Value = 7.00 P-Value = **0.002** DF = 4
Both use Pooled StDev = 0.3198

5. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at -20 ° C (oleuropein)

Sample N Mean StDev SE Mean
1 3 2.5900 0.0500 0.029
2 3 2.7400 0.0800 0.046

Difference = mu (1) - mu (2)
Estimate for difference: -0.1500
95% CI for difference: (-0.3541, 0.0541)
T-Test of difference = 0 (vs not =): T-Value = -2.75 P-Value = **0.051** DF = 4
Both use Pooled StDev = 0.0667

6. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at 4 °C (oleuropein)

Sample N Mean StDev SE Mean
1 3 2.5900 0.0500 0.029
2 3 2.0600 0.0200 0.012

Difference = mu (1) - mu (2)
Estimate for difference: 0.5300
95% CI for difference: (0.4135, 0.6465)
T-Test of difference = 0 (vs not =): T-Value = 17.05 P-Value = **0.000** DF = 4
Both use Pooled StDev = 0.0381

7. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at 20 ° C (oleuropein)

Sample N Mean StDev SE Mean
1 3 3.940 0.190 0.11
2 3 2.9100 0.0200 0.012

Difference = mu (1) - mu (2)

Estimate for difference: 1.030
95% CI for difference: (0.617, 1.443)
T-Test of difference = 0 (vs not =): T-Value = 9.34 P-Value = **0.001** DF = 4
Both use Pooled StDev = 0.1351

8. Two-Sample t-Test and CI for olive leaf extract using 60% ethanol at 30 °C (oleuropein)

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 3.940 | 0.190 | 0.11 |
| 2 | 3 | 1.5100 | 0.0870 | 0.050 |

Difference = mu (1) - mu (2)
Estimate for difference: 2.430
95% CI for difference: (1.978, 2.882)
T-Test of difference = 0 (vs not =): T-Value = 20.14 P-Value = **0.000** DF = 4
Both use Pooled StDev = 0.1478

## Appendix 4. 7 :Two Sample t-Test Results For 80% Ethanol Based Olive Leaf Extracts

1. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at minus 20 degree C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 6.280 | 0.230 | 0.13 |
| 2 | 3 | 6.6400 | 0.0700 | 0.040 |

Difference = mu (1) - mu (2)
Estimate for difference: -0.360
95% CI for difference: (-0.880, 0.160)
T-Test of difference = 0 (vs not =): T-Value = -2.59 P-Value = **0.060** DF = 4
Both use Pooled StDev = 0.1700

2. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at 4 degree C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 6.380 | 0.230 | 0.13 |
| 2 | 3 | 6.4570 | 0.0100 | 0.0058 |

Difference = mu (1) - mu (2)
Estimate for difference: -0.077
95% CI for difference: (-0.575, 0.421)
T-Test of difference = 0 (vs not =): T-Value = -0.58 P-Value = **0.593** DF = 4
Both use Pooled StDev = 0.1628

3. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at 20 degree C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 6.441 | 0.230 | 0.13 |
| 2 | 3 | 4.473 | 0.160 | 0.092 |

Difference = mu (1) - mu (2)
Estimate for difference: 1.968
95% CI for difference: (1.362, 2.574)
T-Test of difference = 0 (vs not =): T-Value = 12.17 P-Value = **0.000** DF = 4
Both use Pooled StDev = 0.1981

4. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at 30 degree C

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|

1 3 6.441 0.230 0.13
2 3 4.773 0.160 0.092

Difference = mu (1) - mu (2)
Estimate for difference: 1.668
95% CI for difference: (1.062, 2.274)
T-Test of difference = 0 (vs not =): T-Value = 10.31 P-Value = **0.000** DF = 4
Both use Pooled StDev = 0.1981

5. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at minus 20 degree C (oleuropein)

Sample N Mean StDev SE Mean
1 3 5.770 0.370 0.21
2 3 5.680 0.260 0.15

Difference = mu (1) - mu (2)
Estimate for difference: 0.090
95% CI for difference: (-0.888, 1.068)
T-Test of difference = 0 (vs not =): T-Value = 0.34 P-Value = 0.748 DF = 4
Both use Pooled StDev = 0.3198

6. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at 4 degree C (oleuropein)

Sample N Mean StDev SE Mean
1 3 5.770 0.370 0.21
2 3 2.060 0.260 0.15

Difference = mu (1) - mu (2)
Estimate for difference: 3.710
95% CI for difference: (2.732, 4.688)
T-Test of difference = 0 (vs not =): T-Value = 14.21 P-Value = 0.000 DF = 4
Both use Pooled StDev = 0.3198

7. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at 20 degree C (oleuropein)

Sample N Mean StDev SE Mean
1 3 6.540 0.370 0.21

2 3 4.660 0.160 0.092

Difference = mu (1) - mu (2)
Estimate for difference: 1.880
95% CI for difference: (1.008, 2.752)
T-Test of difference = 0 (vs not =): T-Value = 8.08 P-Value = **0.001** DF = 4
Both use Pooled StDev = 0.2850

8. Two-Sample t-Test and CI for olive leaf extract using 80% ethanol at 30 degree C (oleuropein)

| Sample | N | Mean | StDev | SE Mean |
|---|---|---|---|---|
| 1 | 3 | 6.540 | 0.370 | 0.21 |
| 2 | 3 | 4.690 | 0.260 | 0.15 |

Difference = mu (1) - mu (2)
Estimate for difference: 1.850
95% CI for difference: (0.872, 2.828)
T-Test of difference = 0 (vs not =): T-Value = 7.09 P-Value = **0.002** DF = 4
Both use Pooled StDev = 0.3198

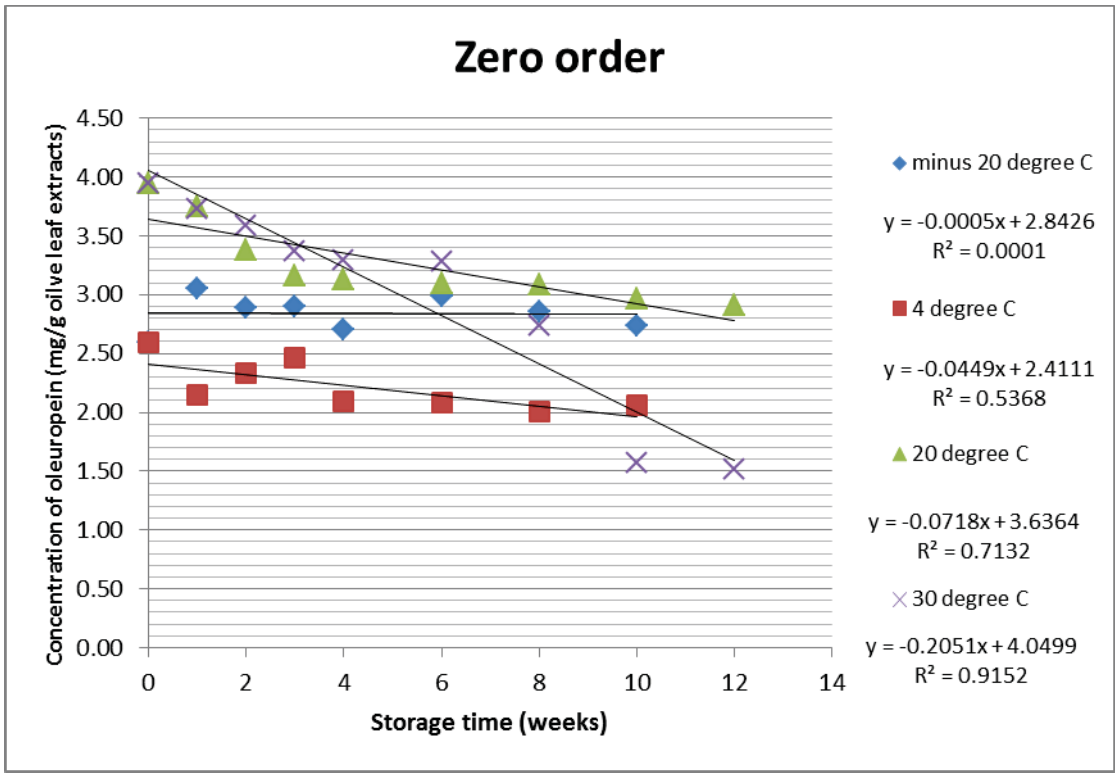
## Appendix 4. 8: Reaction Kinetics

The concentration of oleuropein of 60% ethanol based olive leaf extract stored at four temperatures

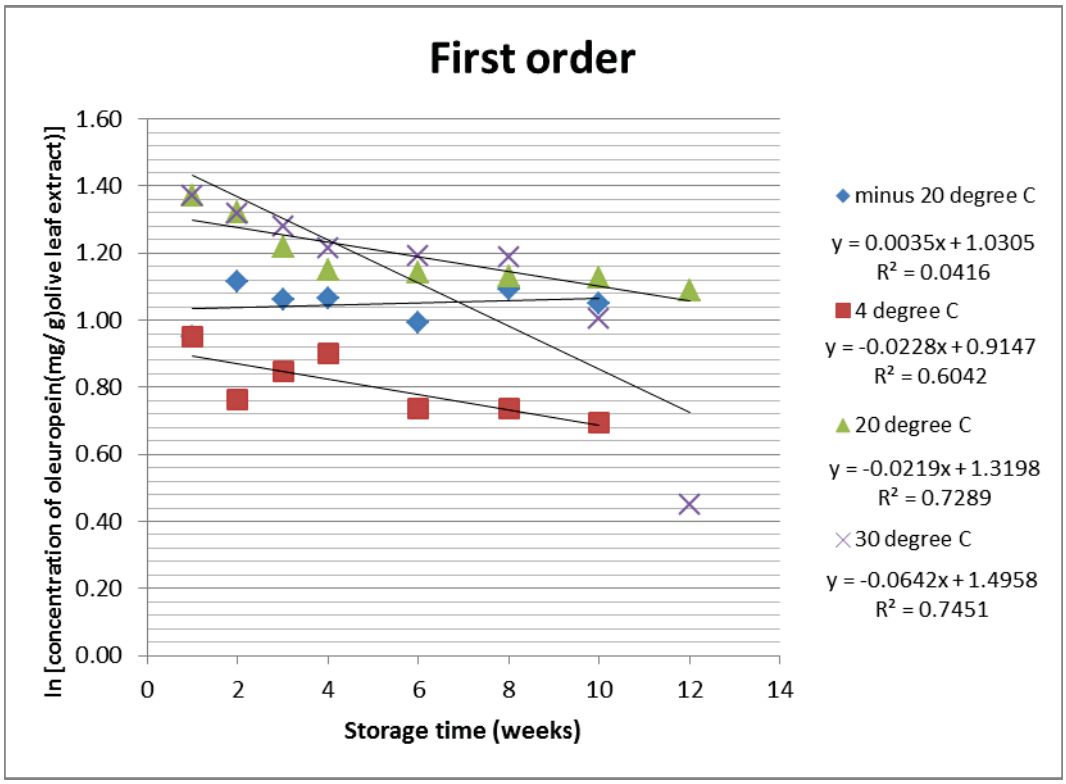
| Storage time | The concentration of oleuropein | The concentration of oleuropein | The concentration of oleuropein | The concentration of oleuropein |
|---|---|---|---|---|
| Weeks | mg/g extracts | mg/g extracts | mg/g extracts | mg/g extracts |
| | -20 ° C | 4 °C | 20 ° C | 30 ° C |
| 0 | 2.59 | 2.59 | 3.94 | 3.94 |
| 1 | 3.05 | 2.15 | 3.75 | 3.73 |
| 2 | 2.89 | 2.33 | 3.38 | 3.59 |
| 3 | 2.90 | 2.46 | 3.16 | 3.37 |
| 4 | 2.70 | 2.09 | 3.13 | 3.29 |
| 6 | 2.98 | 2.08 | 3.10 | 3.28 |
| 8 | 2.86 | 2.00 | 3.09 | 2.73 |
| 10 | 2.74 | 2.06 | 2.97 | 1.57 |
| 12 | -- | -- | 2.91 | 1.51 |

1. Zero order

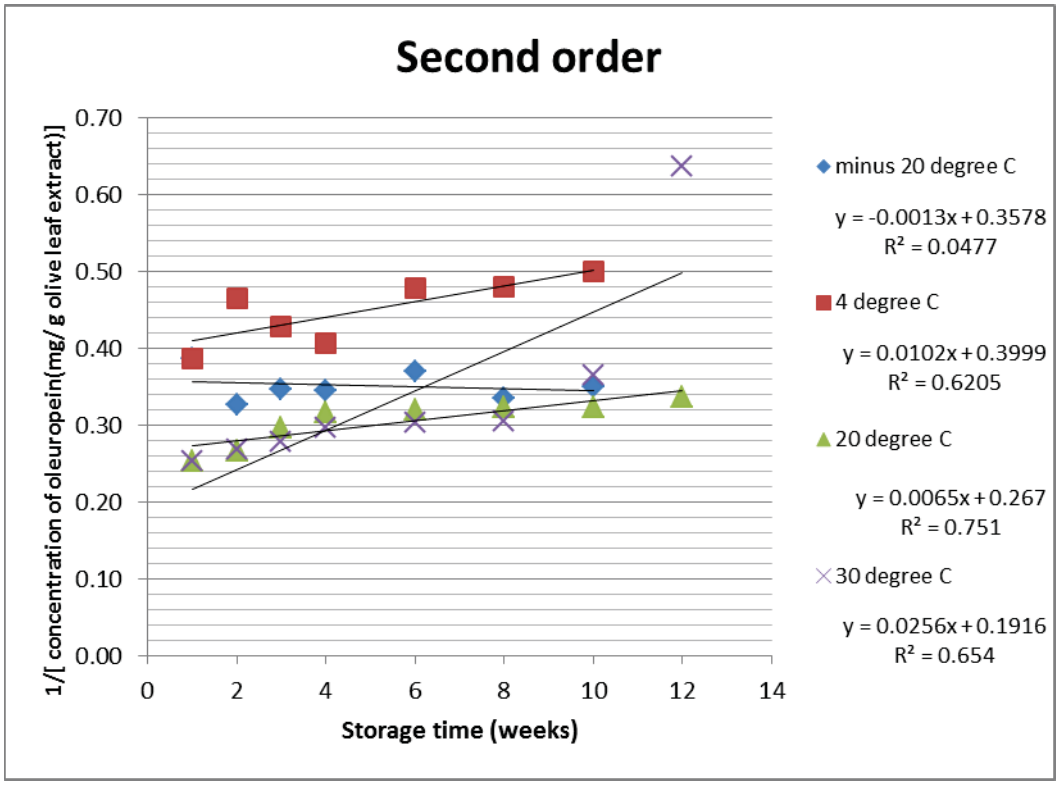
Plot [A] against storage time



2. First order
   Plot ln [A] against storage time



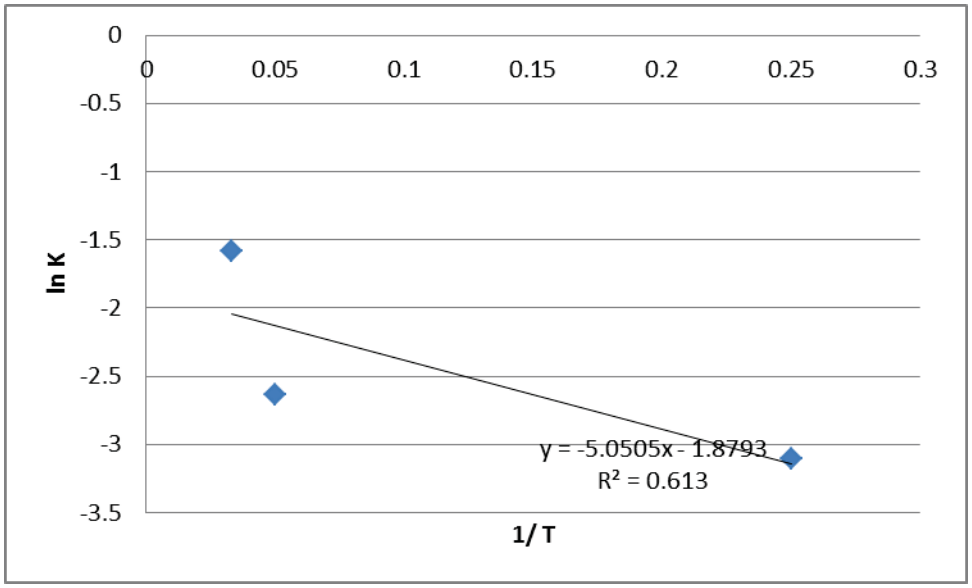

3. Second order
   Plot 1/[A] against storage time




Summary of k values and R square for 60% ethanol based olive leaf extracts

| Condition/ Reaction order | Zero order | | | First order | | | Second order | | |
|---|---|---|---|---|---|---|---|---|---|
| | K | Constant | R square | K | Constant | R square | K | Constant | R square |
| -20°C | 0.00 | 2.84 | 0.00 | 0.00 | 1.03 | 0.04 | 0.00 | 0.35 | 0.05 |
| 4 ° C | 0.04 | 2.41 | 0.54 | 0.02 | 0.91 | 0.60 | 0.01 | 0.40 | 0.62 |
| 20 °C | 0.07 | 3.64 | 0.71 | 0.02 | 1.32 | 0.73 | 0.01 | 0.27 | 0.75 |
| 30 ° C | 0.21 | 4.05 | 0.92 | 0.06 | 1.50 | 0.75 | 0.03 | 0.19 | 0.65 |

Plot lnk against 1/T



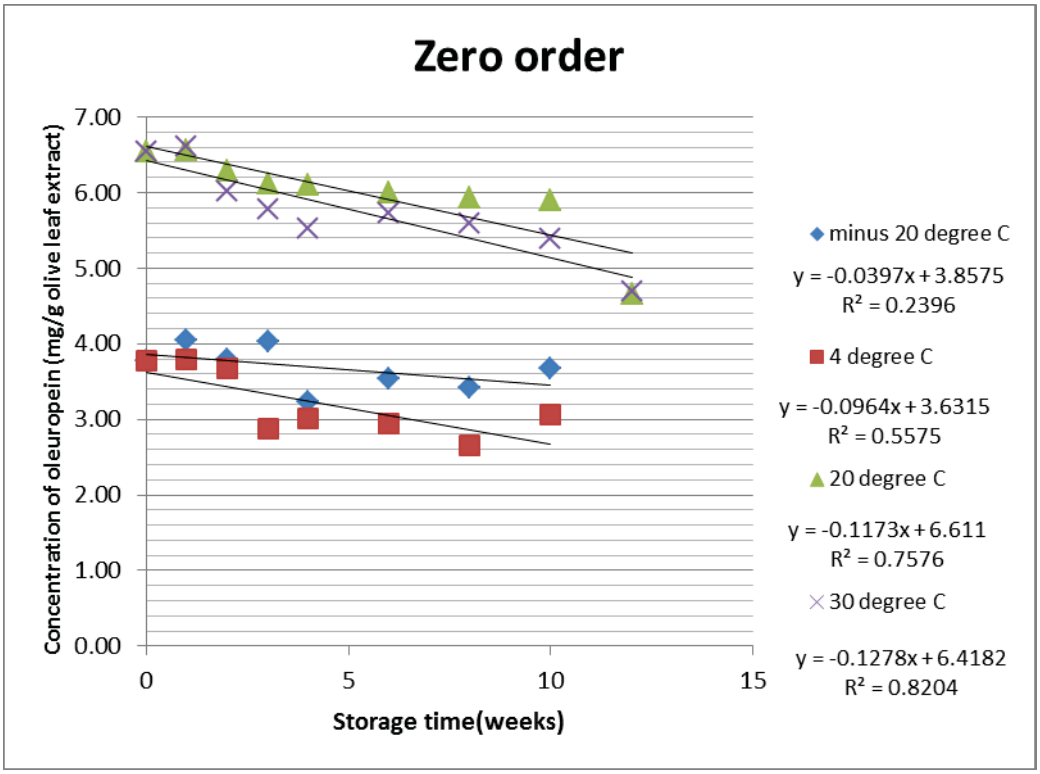

Slope =-$E_A$/R

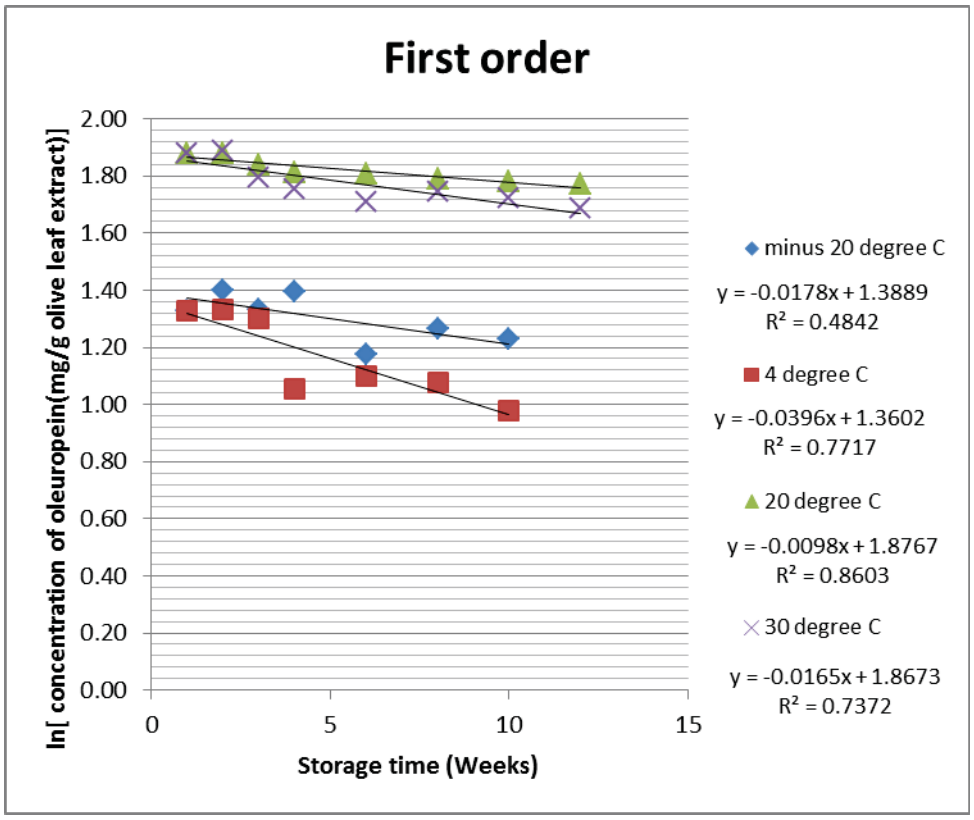
R is the universal gas constant (1.9872 cal/(mol K) or 8.3144 J/(mol K))

$E_A$=5.0505x8.3144=41.9918 J/(mol K)

The concentration of oleuropein of 80% ethanol based olive leaf extract stored at four temperatures

| Storage time | The concentration of oleuropein | The concentration of oleuropein | The concentration of oleuropein | The concentration of oleuropein |
|---|---|---|---|---|
| Weeks | mg/g extracts | mg/g extracts | mg/g extracts | mg/g extracts |
| | -20 °C | 4 °C | 20 °C | 30 °C |
| 0 | 3.77 | 3.77 | 6.54 | 6.54 |
| 1 | 4.05 | 3.79 | 6.55 | 6.61 |
| 2 | 3.79 | 3.68 | 6.29 | 6.02 |
| 3 | 4.03 | 2.87 | 6.13 | 5.78 |
| 4 | 3.24 | 3.00 | 6.10 | 5.52 |
| 6 | 3.54 | 2.94 | 6.00 | 5.73 |
| 8 | 3.41 | 2.66 | 5.93 | 5.59 |
| 10 | 3.68 | 3.06 | 5.89 | 5.40 |
| 12 | | | 4.66 | 4.69 |




First order
ln[ concentration of oleuropein(mg/g olive leaf extract)]
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
5
10
15
Storage time (Weeks)
minus 20 degree C
y = -0.0178x + 1.3889
R² = 0.4842
4 degree C
y = -0.0396x + 1.3602
R² = 0.7717
20 degree C
y = -0.0098x + 1.8767
R² = 0.8603
30 degree C
y = -0.0165x + 1.8673
R² = 0.7372

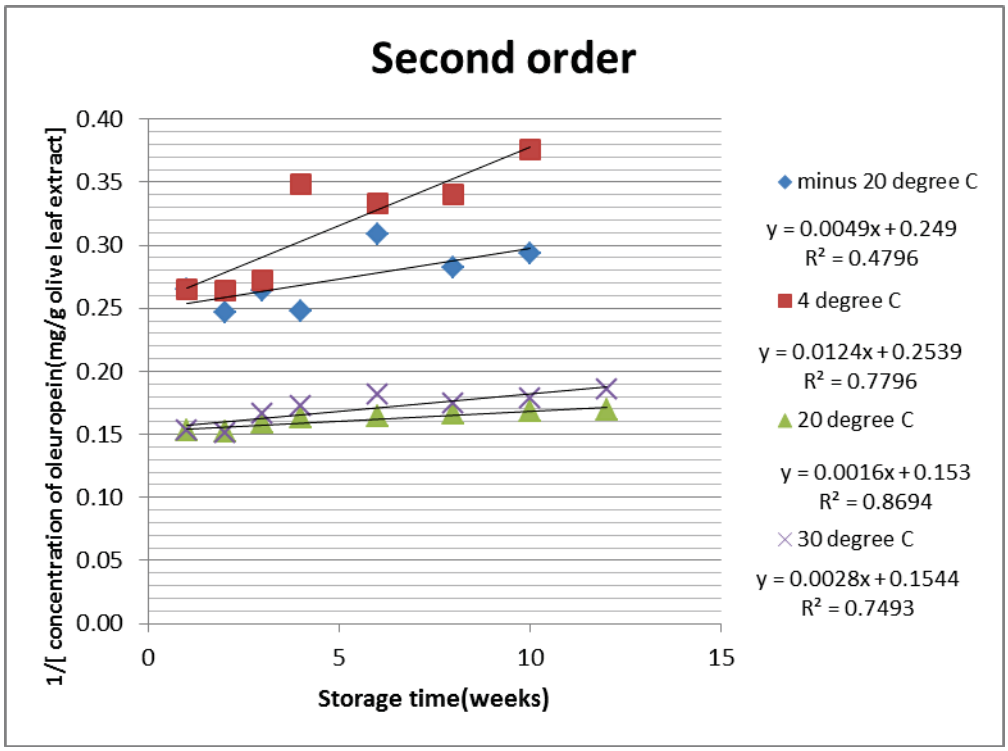
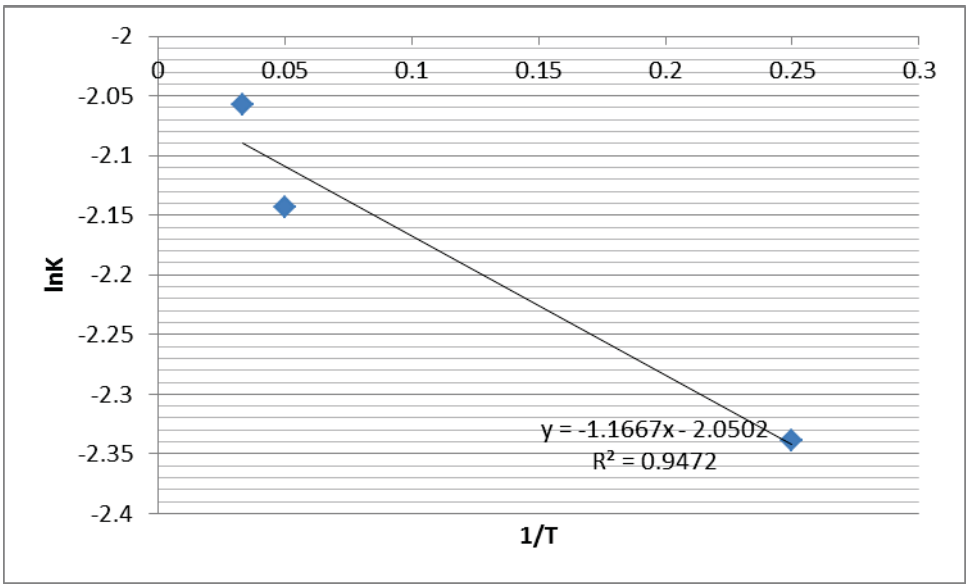

Second order
1/[ concentration of oleuropein(mg/g olive leaf extract]
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
Storage time(weeks)
minus 20 degree C
y = 0.0049x + 0.249
R² = 0.4796
4 degree C
y = 0.0124x + 0.2539
R² = 0.7796
20 degree C
y = 0.0016x + 0.153
R² = 0.8694
30 degree C
y = 0.0028x + 0.1544
R² = 0.7493

Table 4.2.4 Summary of k values and R square for 80% ethanol based olive leaf extracts

| Condition/ Reaction order | Zero order | | | First order | | | Second order | | |
|---|---|---|---|---|---|---|---|---|---|
| | K | Constant | R square | K | Constant | R square | K | Constant | R square |
| -20°C | 0.04 | 3.86 | 0.24 | | | | | | |
| 4 ° C | 0.10 | 3.63 | 0.56 | 0.04 | 1.36 | 0.77 | 0.01 | 0.25 | 0.78 |
| 20°C | 0.12 | 6.61 | 0.76 | 0.01 | 1.88 | 0.86 | 0.00 | 0.15 | 0.87 |
| 30°C | 0.13 | 6.42 | 0.82 | 0.02 | 1.87 | 0.74 | 0.00 | 0.15 | 0.75 |




Slope =-$E_A$/R

R is the universal gas constant (1.9872 cal/(mol K) or 8.3144 J/(mol K))

$E_A$=1.1667x8.3144=9.7004 J/(mol K)