

Free research proposal on efficacy of olive leaves extracts against

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THE OXIDATION OF OLIVE OIL UNDER MICROWAVE HEATING

Chemistry: Research Proposal

Research is proposed to determine whether or not olive leaves are a good source of antioxidants. Reports of experiments to determine the effectiveness of natural plant extracts including olive leaf extracts have found that to be the case. The olive tree (*Olea europaea*) leaves are attractive because they “ contain secoiridoids (oleuropein, ligstrosid, dimethyloleuropein, and oleoside), flavonoids (apigenins, kaempferol, leteolin), as well as phenolic compounds (caffeic acid, tyrosol, hydroxytyrosol).” (Rafiee et al., 2012, p. 1498) High content of antioxidant properties and total phenols have been determined from olive leaf extractions. (Rafiee et al., 2012;) Rafiee (et al., 2012, p. 1507) reported their findings that “ methanol extracts were the most effective in retardation of oxidation, a phenomenon that can be explained by their high phenolic contents.” Their research compared three solvents (80% methanol, acetone and water) for olive leaf extractions. Methanol gave the best results, acetone did not give very good result and water extracts were found to be ineffective. Rafiee (et al., 2012) explained that the dicarboxylic phenolic content of acetone and the good solubility in oil were the reasons that acetone exhibited more inhibition than the water extractions.

Oxidation of food products is caused by free radicals that produce negative effects such as changes in appearance, consistency, taste, aroma and consistency. In order to prevent adverse changes in food due to oxidation synthetic antioxidants have been used (BHA, BHT, and TBHQ). Unfortunately

the synthetic antioxidants are not satisfactory for preventing instability problems in high oil and fat content foods because some are temperature sensitive and can be volatile. Synthetic compounds have also been linked to negative health issues such as cancer. Some natural plant extracts have antioxidant properties due to their high concentration of phenols. Therefore research is necessary to find the most efficient natural material to use coupled with the most efficient extraction solvents and processes. The results are judged by measuring total phenolic content and the total antioxidant capacity.

Extraction techniques for natural plant materials are important for medicinal research and food industry uses. Research of a variety of natural plant materials extracted with different solvents have resulted in interesting data on faba beans cotton seeds, volatile and essential oils and a large range of other plant compounds and a variety of extraction solutions. (Mandal et al., 2007; Kaufman and Christen, 2002) Kaufmann and Christen (2002) compared two extraction methodologies on a variety of plant materials (a) microwave assisted extraction (MAE) and (b) pressurised solvent extraction. They found that both methods were more efficient than traditional methods resulting in “ reduced solvent consumption and shorter extraction times.” (Kaufman and Christen, 2002, p. 105) Mandal, Mohan and Hemalatha (2007) used the MAE strategy for extractions related to medicinal plants. They also found the technique was more effective than conventional methods. The objective of their research is to isolate and identify “ naturally occurring substances. Chemical analysis of extracts from plant material will play a central role in the development and modernization of herbal medicine.”

(Mandal et al., 2007, p. 17) The types of plant material they used ranged from the essential oil of cumin (cuminum cyminum), dried apple pomace (pectin), green tea leaves (polyphenols and caffeine) and tobacco leaves (solanesol). (Please note that the analyte for the plant material is in parenthesis behind the appropriate plant material). The effectiveness of seven solvents was compared in the experiment acetone, acetonitrile, ethanol, hexane, methanol, 2-propanol and water. (Mandal et al., 2007, p. 17)

Rafiee (et al., 2012) have conducted research using the MAE technique for extraction in order to measure the antioxidant effect on sunflower oil using olive leaves. Japón-Luján and Luque de Castro conducted research to determine the amount of biophenols (BPs) available from olive tree leaves and small branches from olive trees. They determined that simultaneous extraction of the two materials (olive tree leaves and small branches) offers a high variety of BPs. When the extractions were done separately the leaves produced “ extracts richer in oleuropein and verbacoside” whereas the small branches produced “ an extract rich in tyrosol, α -taxifolin, and hydroxytyrosol.” (Japón-Luján and Luque de Castro, 2007, p. 4584)

Equations and how to compare measurements

Below are the necessary equations using the results of measurement of extracted samples and blanks (for control) in order to analyze the data.

For determination of Total Phenolic Content

Absorbance at 760 nm will be used to create a calibration curve so that the linear equation to calculate the phenolic content can be written.

$$(1)y = (m) x + b,$$

Where y = absorbance, m = slope, b = intercept, and x = concentration ($\mu\text{g ml}^{-1}$)

Reducing power assay

The absorbance (at 700 nm) of the mixture containing the supernatant of extraction solution, distilled water, and FeCl_3 solution will be used to assay the reducing power at different concentrations. High absorbance directly correlates with high reducing power.

Trial antioxidant capacity

A higher absorbance value directly correlates with higher antioxidant activity.

Oxidative Stability

The peroxide value (PV) will be used to calculate the antioxidant activity according to the following equation.

$$(2)100 - \left(\frac{\text{PV sample}}{\text{PV control}} \times 100\right) = \text{Antioxidant activity}$$

Purpose Statement

Previous research in our laboratory demonstrated that olive leaves could be a good source of antioxidants. Microwave Assisted Extraction (MAE) has been successfully used to collect extract from olive leaves with a high content of antioxidant properties and total phenols. (Rafie et al., 2012) The proposed research will be based upon previously published research using the MAE methodology. The project will also examine the possible application of olive leaf extracts to inhibit olive oil oxidation and quality deterioration during

microwave heating. A comparison will be made between using (a) olive oil and (b) olive oil with olive leaf extract to inhibit olive oil oxidation and deterioration during microwave heating. The next comparison will be between (a) a food (bread) with olive oil and (b) a food with olive oil and olive leaf extract. A comparison will be done between water and methanol extracts in order to understand the better extract to use.

Aims/Objectives

The main objective of the proposed research is to evaluate whether olive leaf extract can reduce oil oxidation under microwave heating. In other words the experiment will be performed in order to determine if olive leaf extract can be used as a natural source of antioxidant. A good methodology for extracting antioxidant properties and phenols from olive leaves will be established. Another aim of the experiment is to evaluate the best extraction solvent to use: (a) water or (b) methanol. Determine the best amount of olive leaf power to mix with each solvent before the solution is irradiated. Mandal (et al., 2007., p. 17) noted enthusiastically in the conclusion to their article in *Pharmacognosy Reviews* that “ The majority of extraction procedures for the determination of plant metabolites are developed in such a way that the final extract introduced into the GC (gas chromatography) or the HPLC (high performance liquid chromatography) columns contains only the analytes with all interferences removed. This is one area where conventional techniques have spelled utter disaster.”

Methods approach

A literature review will be carried out to collect articles on the experimental data for similar experiments from peer reviewed journals. In this way the researchers can evaluate what studies have been done and what the studies may be lacking. The main experimental methodologies will be studied to learn the best way to approach the MAE. The published research will be evaluated to determine the range of solvents used and if water or methanol has been shown to work better. Each trial will be replicated twice in order to determine the reliability of the method. Resulting measurements will be read three times so that a determination can be made of the accuracy of the experimental method.

Experimental Methodology

In a preliminary literature review an analytical process for olive leaf extracts using a modified home microwave that is suitable for the proposed research was found. Rafiee, Jafari, Almami, and Khomeiri (2012) measured the antioxidant effect on sunflower seed oil using a MAE . The proposed research will use virgin olive oil in place of the sunflower seed oil. The following methodology has been adapted from their paper.

Olive leaf preparation.

Olive leaves will be dried in the open air, protected from direct sunlight until the leaves dry completely. Grind to a powder and pass through a 60-mesh sieve.

Microwave modification.

A household microwave will be modified for laboratory use. A magnetic stirrer will be added to the bottom of the oven, below where the boiling flask will be placed. Three other elements will be added to adapt the microwave oven for the experiment (a) water condenser for the water condensation from the boiling water in the flask, (b) a temperature sensor and (b) time controlling device. (Adapted from Rafiee et al., 2012)

Procedure.

The dried olive leaves need to be divided so enough sample is available to mix with two different solvents for two trials each. That means enough olive leaves need to be available for at least 4 trials. Extra will be needed in case more than two trials for each solvent will be needed. Olive leaf powder will be mixed with 80% methanol for two trials and with water for two trials. The resulting solution will be placed in the 250 ml boiling flask, and then irradiated by the microwave with stirring.

Pre-set irradiation procedure for extraction.

For each trial the solution will be irradiated 3 times or until the desired temperature is reached. Afterwards 3 seconds heating and then turn off the microwave for 15 seconds to allow cooling. (Do not let solution super-boil.) Continue irradiation procedure for 15 minutes. Each extract will be filtered (with the Whatman filter paper) so that fine particles can be separated out. The filtrates from the solution with methanol need to be evaporated at 40° C to dryness using a rotary evaporator. The filtrate using water as the solvent

will be freeze dried and after drying store at 4° C until needed for the determining the total phenolic content.

Total phenolic content determination.

20 µl of each extract was measured and mixed with 1. 16 ml of distilled water and 100 µl Folin-Ciocalteu reagent. And then 300 µl of 20% Na₂CO₃ after 1 minute, up to 8 minutes. Each of the mixtures will then be incubated in a shaking incubator for 30 minutes at 40° C. The absorbance for each sample will be determined at 760 nm. The results will be used to create a calibration curve to determine the appropriate linear equation.

DPPH Radical Scavenging Activity

In order to determine DPPH radical scavenging activity each sample and a blank will be evaluated by mixing 1 mM methanol solution DPPH with 3 ml extract solution (50 – 1000 µg ml⁻¹) Thirty minutes of incubation in darkened room at room temperature. Absorbance at 517 nm will be measured. Total antioxidant capacity.

An assay for total antioxidant capacity will be carried out using the sample for a reduction reaction of Mo(VI) to Mo(V). Phosphate/Mo(V), a green colored complex at an acidic pH. Lab Procedure:

Sample solution – 50-1000 µg dried extract in 1 ml of methanol solvent.

Reagent solution – 0. 6 M sulphuric acid, 28 mM sodium phosphate and 4mM of ammonium molybdate

Blank – 1 ml reagentsolution with solvent. (one for each solvent)

Use the same procedure for samples in the water solvent. Take a 0. 1 ml

aliquot of the sample solution to mix in an Eppendorf tube with 1 ml of reagent. Cap the tubes and incubate the samples and blanks for 90 minutes at 95° C. Cool. Measure absorbance at 695 nm of the blanks and the samples. Absorbance correlates with the amount of antioxidant activity. Higher absorbance values correlate with higher antioxidant activity. (Rafie et al., 2012)

Reducing Power Assay

(Reaction to determine the iron (III) reduction capacity of extracts.) Mix the dried extract with chosen solvent, 2.5 ml of phosphate buffer, 2.5 ml of potassium ferriocyanide; then the samples and blank mixtures will be incubated at 50° C for thirty minutes. Next 2.5 ml trichloroacetic acid was added to each mixture and each was centrifuged ten minutes at 1650g. A mixture will be made of supernatant solution (2.5 ml), distilled water (2.5 ml) and FeCl₃ (0.5 ml of 1 g l⁻¹) and then measured absorbance at (700 nm) of samples and blank will be carried out.

Total antioxidant capacity.

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Sample solution – 50-1000 µg dried extract in 1 ml of methanol solvent.

Reagent solution – 0.6 M sulphuric acid, 28 mM sodium phosphate and 4mM of ammonium molybdate

Blank – 1 ml reagent solution with solvent. (one for each solvent)

Use the same procedure for samples in the water solvent. Take a 0.1 ml aliquot of the sample solution to mix in an Eppendorf tube with 1 ml of reagent. Cap the tubes and incubate the samples and blanks for 90 minutes at 95° C. Cool. Measure absorbance at 695 nm of the blanks and the samples. Absorbance correlates with the amount of antioxidant activity. Higher absorbance values correlate with higher antioxidant activity. (Rafie et al., 2012)

Oxidative stability

Mix each of the following amounts, 200, 500 and 1,000 ppm concentrations, of olive leaf extracts with the virgin olive oil; prepare blanks. Store in a 70° C oven for 12 days. And then analyze the mixtures and blank for Peroxide Value (PV) with the AOAC method.

Statistical analysis. Two trials will be done for each sample for each procedure. Analysis of Variance will be calculated in order to determine significant deviance that will need to be taken into account when evaluating the results.

Materials

Virgin olive oil

Olive tree leaves

Olive leaf powder from dried olive leaves

80% methanol (solvent)

Distilled water (enough for 2 uses, for solvent, cooling and determining the total phenolic content)

Folin-Ciocalteu reagent

Equipment

60-mesh sieve

Modified household microwave oven (See fig. 1)

Magnetic stirrer

Water condenser

Temperature sensor

Time control device

250 ml boiling flask

Connecting tube

Energy attenuator

Condensing coil

Temperature recorder

Speed controller

Time controller

Wattman No. 1 filter

Freeze dry instrument

Rotary evaporator

Shaking incubator

UV absorbance measurement instrument

Centrifuge

Figure 1. A modified microwave oven adapted for use during the Microwave Assisted Experiment (MAE). Source: Rafiee et al., 2012, p. 1499)

Milestones and timetable

Budget and resources

Total budget amount: 400 Australian Dollars

Reagents and chemicals = 300 Australian Dollars

Flasks and glassware - ! 00 Australian Dollars

Risk to project failure

Super boiling the solution in the microwave would ruin the experiment because the liquid would all be lost to condensation. If measurements are carried out carefully the results will be replicable. If two trials of each sample are not made the statistical analysis cannot be done.

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