*Short Communication*

**Analysis of the nutritional composition of round-leaved mallow (*Malva pusilla*) leaves**

**Supplementary File: Materials and Methods**

***1. Analysis of Lipids and Fatty Acids***

*Lipid Extraction*

Lipids were extracted from *Malva pusilla* following the method of Folch et al. (1957). Chemicals and Reagents needed: Methanol (CH3OH) (Analar, England), chloroform (CHCl3) (Sigma-Aldrich Corp, France), deionized water (Milli-Q, Merk, France), and nitrogen gas (Yateem Oxygen, Bahrain).

The samples with different weights (1 g and 2 g) were added to a combination of 19 ml chloroform: methanol (2:1) and mixed vigorously for 10 minutes. A volume of 4 mL of deionized water was added, and the mixture was mixed once for 10 minutes. Samples were added into a plastic centrifuge tube and centrifuged at 2400 rpm for 20 minutes. The lower phase was transferred into a pre-weight glass tube and dried in a water bath at 37 °C using nitrogen gas until a clear oil appeared at the tip of the tube. Last, the test tubes were weighed again to determine the total lipid content and stored for fatty acid analysis.

*Fatty Acid Methylation*

Chemicals and Reagents: Deionized water was used to prepare the solutions. Sodium hydroxide pellets (NaOH) (Sigma-Aldrich Corp, United Kingdom), methanol (CH3OH) (Analar, England) 14% boron trifluoride-methanol (CH4BF3O) (Aldrich, Sigma-Aldrich, USA), isooctane (CH3C) (Fluka Chemika, Switzerland), sodium chloride (NaCl) (Sigma-Aldrich, Germany), sodium sulphite anhydrous (NaSO3) (Eurostar Scientific LTD, Cheshire, United Kingdom), and methanolic sodium hydroxide (100 ml methanol with 2 g NaOH).

Fatty acids’ methylation was performed according to Ozogul et al. (2012): The full extracted lipids were combined with 1.5 ml of 0.5 M methanolic sodium hydroxide in a test tube, then it was heated for 7 minutes at 100 °C and let to cool down at room temperature. A volume of 2 ml of 14% boron trifluoride-methanol was added, and after 5 minutes of heating at 100°C, the mixture was cooled to 30–40°C. A volume of 1 ml of isoocatane was added and the tube was shaken by a vortex for 30 seconds. A volume of 5 ml of saturated sodium chloride was added and was shaken the tube for 30 seconds after that. The upper isooctane layer that contains fatty acid methyl esters (FAMEs) was removed and placed into another test tube and the bottom layer was then extracted once again with 1 ml of isooctane and shaken for 30 seconds. The two extracted isooctane layers were combined, concentrated to 1 ml, and dried by adding a small amount of anhydrous sodium sulphate under the nitrogen gas.

*Fatty Acid Analysis*

Fatty acid methyl esters (FAMEs) were separated using a fused carbon-silica column (Stabilwax, Crossbond, Carbowax, Polyethylene glycol) that has a length of 30 m, an internal diameter of 0.25mm, 0.25µm film thickness and the temperature range of 40 to 260°C. Oven’s initial temperature was set at 115°C, then held for 2 minutes, ramped 10°C/min to 200°C, held 18.5 minutes, ramped 60°C/min to 245°C, then held for 4 minutes (each sample’s total run time is 30 minutes), the FID temperature was set at 300°C, TCD temperature was set at 150°C, and the injector temperature will be 300°C with 1:2 split ratio. Nitrogen gas was used as a carrier gas at a flow rate of 0.76 ml/min, also the air (450 ml/min) and hydrogen (45 ml/min) were used. The sample injected volume was 5 µl with three pre and post-injection sample washes. The sampling rate used was 12.5 Hz. The FAMEs were recognized by comparing their peak areas against authentic standards (PUFA NO.1 and PUFA NO2.) that were supplied by SUPELCO USA.

***2. Analysis of Minerals***

Materials and instruments: 65% nitric acid (HNO3) (Sigma-Aldrich, Germany), 30% hydrogen peroxide solution (H2O2) (AnalaR, UK), lab microwave digestion system (CEM, United States of America), membrane filter with a pore size of 0.45μm (Nalgene, New York), standard solutions of Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn (Sigma-Aldrich, Switzerland) and inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (PerkinElmer, USA).

Preparation of 2% HNO3: To prepare 500 ml of 2% HNO3, 15.38 ml of 65% HNO3 was mixed with 484.62 ml of water, then the mixture was poured into a tightly sealed clean dry storage glass bottle and was stored in the fume hood.

*Sample digestion by MARS6*

Malva Samples will be digested and analyzed in triplicates. First, an amount of 0.5 g of sample was taken and added into microwave tubes (EasyPrep iWave) followed by the addition of 1 ml of 30% hydrogen peroxide and 5 ml of 65% nitric acid. Then, the microwave tubes were transferred to the MARS6 microwave digestion system to begin the digestion process. After one hour, the digestion process was over, the mixture was filtered using a membrane filter with a pore size of 0.45 μm and diluted in volumetric flasks with 2% HNO3 to a final volume of 25 ml using a Pasteur pipette with a bulb.

*Sample preparation for mineral determination using ICP-OES*

Tubes used for the ICP-OES were first rinsed with 2% HNO3. Samples were diluted 10 times by mixing 0.5 ml from each sample with 9.5 mL of 2% HNO3. The five standards for each mineral will be prepared using stock solutions of Ca, Cu, Fe, Zn, Mg, Mn, Na, and K (1000 mg/L). Samples will be analyzed using ICP-OES at the following wavelengths (nm) for each mineral: Ca - 317.933 nm, Cu - 327.393 nm, Fe - 238.204 nm, Zn - 206.200 nm, Na - 589.592 nm, K - 766.490 nm, Mn - 257.610 nm, and Mg - 285.213 nm.

***References***

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry*, *226*(1), 497–509.

Ozogul, Y., Simşek, A., Balikçi, E., & Kenar, M. (2012). The effects of extraction methods on the contents of fatty acids, especially EPA and DHA in marine lipids. *International journal of food sciences and nutrition*, *63*(3), 326–331. https://doi.org/10.3109/09637486.2011.627844