Ultrasound-Assisted Extraction of Polyphenolic Contents and Acid Hydrolysis of Flavonoid Glycosides from Oil Palm (Elaeis guineensis Jacq.) Leaf: Optimization and Correlation with Free Radical Scavenging Activity

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Mohamad Shazeli Che Zain, Nurul Azani Jakariah, Jen Xen Yeoh, Soo Yee Lee, Khozirah Shaari

Abstract:

Malaysia is ranked as the second largest oil palm producer in the world after Indonesia. This leads to the generation of large quantities of oil palm (Elaeis guineensis Jacq.) leaves (OPLs) annually, considered an underutilized oil palm biomass with low economical value. The present study aimed to study the effects of several parameters of ultrasound-assisted extraction (UAE) and the acid hydrolysis of phenolic compounds from OPLs using the single factor experimental approach. The effects of different solvents (hexane, ethyl acetate, 1:1 methanol?ethyl acetate, absolute methanol, and 4:1 methanol?water), solid?liquid ratios (1:20, 1:40, 1:50, 1:60, and 1:70), times (0.5, 1, 2, 3, and 5 h), and temperatures (25, 30, 40, 60, and 70 °C) were investigated for UAE. Moreover, the effects of acid concentration (0.2, 1, 2, 4, 6, and 12 M), incubation time (10, 30, 45, 60, and 90 min), and incubation temperature (65, 75, 85, 90, and 95 °C) on the acid hydrolysis of flavonoid glycosides were also determined. The results revealed that some of the tested parameters had prominent effects on the total phenolic (TPC) and total flavonoid (TFC) contents, as well as the DPPH free radical scavenging activity recovered from the OPLs. The optimal UAE conditions were determined to be 0.5 h at 25 °C using 4:1 methanol?water and 1:50 solid?liquid ratio, producing OPL extracts with TPC and TFC at 335.30 and 60.67 milligrams quercetin equivalents per gram of extract (mg QCE/g extract), respectively, and DPPH free radical scavenging activity at 94.06%. The phenolics present in OPLs were optimally hydrolysed using 6 M hydrochloric acid with an incubation period of 45 min at 95 °C with TPC, TFC, and DPPH free radical scavenging activity at 126.33 milligrams gallic acid equivalents per gram of extract (mg GAE/g extract), 36.08 mg QCE/g, and 54.88%, respectively. Moreover, acid hydrolysis managed to optimally recover the total apigenin content (TAC), total luteolin content (TLC), and total flavonoid C-glycoside content (TFCGC) with values of 79.12 micrograms vitexin equivalents per milligram of extract (µg VE/mg extract), 20.97 micrograms orientin equivalents per milligram of extract (µg OE/mg extract), and 100.09 µg/mg, respectively. Additionally, there were significant correlations between the polyphenolic compounds, flavonoid C-glycosides, and antioxidant activity for all parameters based on the Pearson correlation analysis. This indicates that OPLs have potential as a natural source of phenolic compounds, especially flavonoid C-glycosides, with beneficial free radical scavenging activity that can be incorporated in food and pharmaceutical products.

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Abstract: Malaysia is ranked as the second largest oil palm producer in the world after Indonesia. This leads to the generation of large quantities of oil palm (*Elaeis guineensis* Jacq.) leaves (OPLs) annually, considered an underutilized oil palm biomass with low economical value. The present study aimed to study the effects of several parameters of ultrasound-assisted extraction (UAE) and the acid hydrolysis of phenolic compounds from OPLs using the single factor experimental approach. The effects of different solvents (hexane, ethyl acetate, 1:1 methanol–ethyl acetate, absolute methanol, and 4:1 methanol–water), solid–liquid ratios (1:20, 1:40, 1:50, 1:60, and 1:70), times (0.5, 1, 2, 3, and 5 h), and temperatures (25, 30, 40, 60, and 70 °C) were investigated for UAE. Moreover, the effects of acid concentration (0.2, 1, 2, 4, 6, and 12 M), incubation time (10, 30, 45, 60, and 90 min), and incubation temperature (65, 75, 85, 90, and 95 °C) on the acid hydrolysis of flavonoid glycosides were also determined. The results revealed that some of the tested parameters had prominent effects on the total phenolic (TPC) and total flavonoid (TFC) contents, as well as the DPPH free radical scavenging activity recovered from the OPLs. The optimal UAE conditions were determined to be 0.5 h at 25 °C using 4:1 methanol–water and 1:50 solid–liquid ratio, producing OPL extracts with TPC and TFC at 335.30 and 60.67 milligrams quercetin equivalents per gram of extract (mg QCE/g extract), respectively, and DPPH free radical scavenging activity at 94.06%. The phenolics present in OPLs were optimally hydrolysed using 6 M hydrochloric acid with an incubation period of 45 min at 95 °C with TPC, TFC, and DPPH free radical scavenging activity at 126.33 milligrams gallic acid equivalents per gram of extract (mg GAE/g extract), 36.08 mg QCE/g, and 54.88%, respectively. Moreover, acid hydrolysis managed to optimally recover the total apigenin content (TAC), total luteolin content (TLC), and total flavonoid *C*-glycoside content (TFCGC) with values of 79.12 micrograms vitexin equivalents per milligram of extract (μg VE/mg extract), 20.97 micrograms orientin equivalents per milligram of extract (μg OE/mg extract), and 100.09 μg/mg, respectively. Additionally, there were significant correlations between the polyphenolic compounds, flavonoid *C*-glycosides, and antioxidant activity for all parameters based on the Pearson correlation analysis. This indicates that OPLs have potential as a natural source of phenolic compounds, especially flavonoid *C*-glycosides, with beneficial free radical scavenging activity that can be incorporated in food and pharmaceutical products.

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1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a major commodity in many countries, including Malaysia, which is ranked among the top producers of palm oil. In the wake of large-scale cultivation, a huge amount of agricultural waste material is produced. Based on the National strategy, from a supply-side perspective, Malaysia's palm oil industry is anticipated to produce approximately 100 million dry tonnes of solid biomass by 2020. This includes the mesocarp fibres (MFs), empty fruit bunches (EFBs), palm kernel shells (PKSs), oil palm leaves (OPLs), oil palm trunks (OPTs), and oil palm fronds (OPFs) [1]. In fact, a total of 7.1 million tonnes per year of pruned fronds are produced in 2017–2020 [2]. This amount of waste material continually produced from the oil palm industry presents a great challenge to agricultural waste and environmental management. The OPLs are considered underutilized in comparison with other oil palm biomasses. However, the low-value OPLs could be exploited for their utilization in the pharmaceutical field due to its high potential as antioxidants, antimicrobials, and disinfectants (wound healing) [3–5]. Previous screenings of the phytochemical contents of OPL extracts uncovered the presence of tannins, alkaloids, steroids, saponins, terpenoids, phenolics, and flavonoids (Figure 1) [6,7].
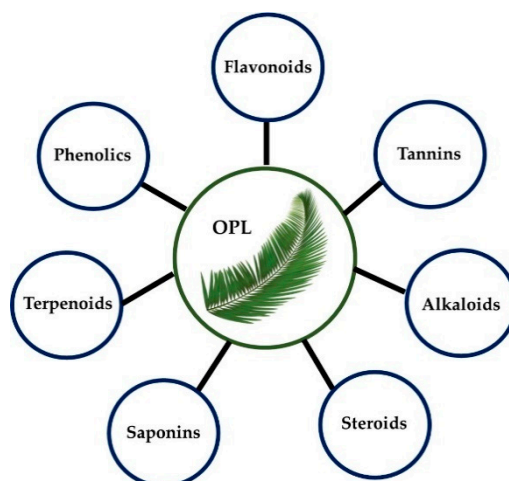
Figure 1. Groups of compounds present in oil palm leaves (OPLs).

Ultrasound-assisted extraction (UAE) is widely recognized as an innovative method of extracting constituents as it is simple, fast, and efficient in comparison with other conventional extraction methods such as maceration, soaking, and Soxhlet extraction [8]. It does not require complex instruments to operate and it can be applied in small or bulk productions in the phytopharmaceutical extraction industry [9]. Previous studies demonstrated that UAE reduces the tendency of phenolic degradation and was a faster method to extract phenolics from different plants and fruits in comparison with other common extraction methods, including solid–liquid and microwave-assisted extractions [10,11].

Previous studies reported that OPLs contain huge amounts of flavonoids, primarily flavonoid *O*-glycosides and *C*-glycosides [12,13]. One of the characteristics of flavonoid *C*-glycosides is high resistance to hydrolysis in comparison with flavonoid *O*-glycosides. The hydrolytic breakdown of glycosides into their component parts can be achieved in multiple ways, including acid hydrolysis, alkaline hydrolysis, and enzymatic hydrolysis. Among these methods, acid hydrolysis is the most commonly used to hydrolyse flavonoid glycosides from various plant matrixes [12,14–16]. Studies have

shown that different conjugates present in plant extracts behaved differently at different acid hydrolysis conditions. Hence, by optimizing the conditions related to the acid hydrolysis process, the optimum amount of flavonoid *C*-glycosides from OPLs could be recovered.

Due to the great diversity of constituents in plants, there is no universal extraction system, and specifically, no acid hydrolysis procedure that is appropriate for the extraction and hydrolysis of all plant constituents. Therefore, the optimization of the extraction and acid hydrolysis process is essential for a precise assessment of phytocompounds from various plant matrices [17]. While the recovery of phytochemicals from plants depends on the extraction conditions, a suitable extraction and acid hydrolysis condition for extracting and hydrolysing the non-resistant flavonoids for the recovery of the resistant flavonoids, specifically flavonoid *C*-glycosides from OPLs, have yet to be discovered. Hence, the purpose of the present study was to determine the effects of the extraction solvent (hexane, ethyl acetate, 1:1 ethyl acetate–methanol, methanol, and 4:1 methanol–water), solid–liquid ratio (1:20, 1:40, 1:50, 1:60, and 1:70), temperature (25, 30, 50, 60, and 70 °C), and time (0.5, 1, 2, 3, and 5 h) towards the extraction of phenolic compounds (total phenolic content—TPC; and total flavonoid content—TFC) and also to investigate their antioxidant capacity (DPPH-scavenging activity) using a single factor experiment approach. Furthermore, the optimization of acid hydrolytic conditions, including the molarity of hydrochloric acid, (0.2, 1, 2, 4, 6, and 12 M), incubation time (10, 30, 45, 60, and 90 min), and incubation temperature (65, 85, 90, and 95 °C) were also investigated for the optimum recovery of flavonoid *C*-glycosides.

2. Materials and Methods

2.1. Chemicals

Sodium carbonate, sodium acetate, Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and quercetin were supplied by Sigma Aldrich (Steinheim, Germany). Hydrochloric acid, and organic solvents (hexane, ethyl acetate, methanol, and ethanol) were provided by R&M Chemicals (Essex, United Kingdom). Aluminium chloride was provided by HmbG Chemicals (Hamburg, Germany). Deionized water was used in this study. Analytical grade water and acetonitrile were bought from Merck (Darmstadt, Germany). Commercial standards of vitexin, orientin, isovitexin and isoorientin with more than 98% purity were purchased from ChemFaces (Wuhan, China).

2.2. Sample Preparation

Mature OPLs were harvested from the University Agricultural Park, Universiti Putra Malaysia (UPM). The leaflets were cleaned, the rachises were removed and cut into smaller pieces (one inch). Oven drying method was used to dehydrate the leaflets. The drying oven was set at 35 °C and the moisture content was monitored regularly until a constant weight was reached. The dried OPLs were ground and sieved to obtain a uniform size of OPL powders. The powders were preserved at −80 °C until further analysis.

2.3. Ultrasound-Assisted Extraction (UAE)

Upon subjecting to UAE, the mixture of OPL powder and extraction solvent was vortexed at 3000 rpm for 30 s. The mixture was sonicated at 40 Hz in a Branson 2510MT Ultrasonic Cleaner (Branson Ultrasonics Corporation, Danbury, CT, USA). The supernatant was obtained after 15 min centrifugation at the speed of 4000 rpm. The concentrated extract was recovered by vacuum evaporation and subsequently subjected to freeze drying to produce a complete dried crude extract. The process was conducted in triplicate for each sample. These extracts were used for phytochemical content analysis and free radical scavenging activity evaluation.

2.3.1. Experimental Design

In the current study, a total of four important extraction parameters, namely solvent, solid–liquid ratio, temperature, and time were studied. The optimum conditions for the extraction of antioxidant phenolic compounds from OPLs were determined using a single factor experiment, whereby one factor was tested at a time while other factors remained unchanged. The optimal conditions were chosen based on the measurements of percentage yield, TPC, TFC, and DPPH-scavenging activity.

Extraction Solvent

To optimize the extraction solvent, the OPL samples were extracted using several extraction solvents, namely hexane, ethyl acetate, 1:1 ethyl acetate–methanol, absolute methanol, and 4:1 methanol–water at a 1:50 solid–liquid ratio, with the extraction time and temperature being fixed at 0.5 h and 25 °C, respectively.

Solid–Liquid Ratio

For the optimization of the solid–liquid ratio, the OPL samples were extracted using the optimal solvent determined in the first step, with the solid–liquid ratio varied from 1:20 to 1:70 (1:20, 1:40, 1:50, 1:60, and 1:70). Extraction time and temperature were fixed at 0.5 h and 25 °C, respectively.

Extraction Time

To optimize the contact time, the OPL samples were extracted using the optimal solvent and solid–liquid ratio determined previously, with the extraction time varied from 0.5 to 5 h (0.5, 1, 2, 3, and 5 h). Extraction temperature was fixed at 25 °C.

Extraction Temperature

To optimize the temperature, the extraction was performed using the optimal factors determined in the previous experiments (extraction solvent, solid–liquid ratio, and extraction time), with the various temperatures ranging from 25 to 70 °C (25, 30, 50, 60, and 70 °C).

2.4. Acid Hydrolysis

To hydrolyse the compounds from OPLs, 5 g of crude OPL extract was dissolved in 50 mL deionized water and mixed with 50 mL hydrochloric acid. The mixture was capped and incubated at the optimum temperature and time using a waterbath shaker (Model BS-10, Jeio Tech Co. Ltd., Seoul, Korea). The mixture was cooled down and mixed with 20 mL methanol, then vortexed at 3000 rpm for 30 s. The mixture was sonicated at 40 Hz for 10 min in a Branson 2510MT Ultrasonic Cleaner. The supernatant was obtained after 15 min centrifugation at the speed of 4000 rpm. The concentrated extract was recovered by vacuum evaporation and subsequently subjected to freeze drying to produce a complete dried hydrolysed extract. The process was conducted in triplicates for each sample. These extracts were used for phytochemical content analysis and free radical scavenging activity evaluation.

2.4.1. Experimental Design

In the current study, a total of three important acid hydrolysis parameters, namely acid concentration, incubation time, and incubation temperature were studied. The optimum conditions for the hydrolysis of flavonoid glycosides from OPLs were also assessed using a single factor experiment. The optimal conditions were determined based on the measurements of total flavonoid content, flavonoid *C*-glycosides, and DPPH free radical scavenging activity.

Acid Molarity

To optimize the acid concentration, the crude OPL extracts were mixed using several acid concentrations ranging from 0.2, 1, 2, 4, 6, to 12 M with the incubation time and temperature fixed at two hours and 85 °C.

Incubation Time

For the optimization of incubation time, the crude OPL extracts were hydrolysed using the hydrochloric acid concentration determined in the previous step, with the incubation times ranging from 10 to 120 min (10, 30, 45, 60, 90, and 120 min). Incubation temperature was fixed at 85 °C.

Incubation Temperature

To optimize the incubation temperature, the crude OPL extracts were hydrolysed using the best acid concentration and incubation time determined previously, with the incubation temperature ranging from 65 to more than 95 °C (65, 75, 85, 90, and >95 °C).

2.5. Yield Determination

After solvent removal, the crude product was reconstituted with 3 mL of solvent. The crude and hydrolysed OPLs were freeze-dried for complete moisture removal. The calculation of the percentage of yield was done as

$$\text{yield \% = actual yield/theoretical yield} \times 100 \qquad (1)$$

whereby yield % is the percentage yield (%), actual yield is weight of crude OPLs after freeze drying (g), and the theoretical yield is the weight of OPL powder (g).

2.6. Total Phenolic Content (TPC) Determination

TPC was evaluated using a Folin–Ciocalteu (F–C) reagent as previously performed by Lee et al. [18] with slight modifications. Firstly, 0.1 mg/mL of sample were prepared by mixing the extracts in methanol. Subsequently, in each well of the 96-well plate, a volume of 120 µL mixture comprising of 20 µL and 100 µL F–C reagent was added and incubated for 5 min. After adding 80 µL of 7.5% sodium carbonate solution, the absorbance was read at 750 nm using a microtiter plate spectrophotometer. Each sample was analysed in triplicate. The values were stated either in milligrams of quercetin equivalents per gram of extract (mg QCE g^{-1} extract) or in milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

2.7. Total Flavonoid Content (TFC) Determination

TFC was conducted using an aluminium chloride complex forming assay as formerly performed by Formagio et al. [19] and Lin and Tang [20] with some modifications. Samples were prepared at 0.1 mg/mL by solubilizing the extracts in methanol. In the 2 mL microcentrifuge tube, the 125 µL sample, 375 µL 95% ethanol, 25 µL 10% aluminium chloride solution, 25 µL sodium acetate solution and 700 µL water were mixed homogenously by 30 s vortex and further incubated for 40 min at 25 °C. Two-hundred microlitres (200 µL) of mixture was transferred into each well of the 96-well plate and the absorbance was read at 415 nm on a microtiter plate spectrophotometer. Each sample was analysed in triplicate. The values were stated in milligrams of quercetin equivalents per gram of extract (mg QCE/g extract).

2.8. Quantitative and Qualitative Analysis of Total Apigenin, Total Luteolin, and Total Flavonoid C-Glycosides

The quantitative analysis of the flavonoid *C*-glycosides of interest was performed via ultra-high-performance liquid chromatography-ultraviolet/photo-diode array (UHPLC–UV/PDA) approach. The analysis was run on a Thermo Scientific Ultimate 3000 equipped with PDA-3000

photodiode array detector and the column compartment was set at 25 °C. The C_{18} reversed phase Hypersil Gold aQ column (1.9 μm particle size and 2.1 mm internal diameter × 100 mm length) (Dionex, Sunnyvale, CA, USA) was used in this study. For the mobile phases, solvent A was a mixture of water with 0.1% formic acid and 0.063% ammonium formate while solvent B consisted of acetonitrile and 0.1% formic acid. The gradient elution was programmed by following the solvent B (%) sequence: 10% for 0–0.6 min, 10–13.5% for 0–13.5 min, 13.5–100% for 13.5–15 min, and 100–10% for 15–16 min. The flow rate was set at 0.40 mL/min and the peak elution was monitored at 270 and 340 nm UV channels. By injecting the reference standards, the peaks were identified and confirmed based on their respective retention times and UV spectra. The 5 mg/mL samples were prepared and filtered before the sample injection. The validated calibration curves displayed good linearity in the ranges of 31–800 μg/mL for orientin and 47–1500 μg/mL for vitexin. The regression curves for orientin and vitexin were $y = 2706.2x − 19,677$ ($R^2 = 0.9999$, $n = 6$) and $y = 534.05x − 6500.5$ ($R^2 = 0.9997$, $n = 6$), respectively, whereby y is the peak area of the compound and x is the concentration of the respective standards. Total apigenin content (TAC) was established as the addition of apigenin derivatives identified in chromatogram, while the total luteolin content (TLC) was obtained as a result of the addition of luteolin derivatives. The sum of TAC and TLC was established as the total flavonoid *C*-glycosides (TFCGC). The results of total apigenin were stated in micrograms of vitexin equivalents per milligram of extract (μg VE/mg extract), while the results of total luteolin were stated in micrograms of orientin equivalents per milligram of extract (μg OE/mg extract).

The qualitative identity of the flavonoid *C*-glycosides of interest was confirmed via the UHPLC–MS analysis. The analysis was performed on a Q-Exactive Focus Orbitrap LC–MS–MS system where the eluent was controlled by electrospray ionization-mass spectrometry (ESI–MS) under negative mode scanned from *m/z* 67.9 to 1000. ESI was performed using a spray voltage of 4.2 kV. Capillary temperature was set at 320 °C, whereas the aux gas heater temperature was set at 0 °C. The data acquisitions were performed in automatic mode at 30 eV collision energy for tandem MS. Thermo Xcalibur Application version 2.2 by Thermo Fisher Scientific (Waltham, MA, USA) was used to conduct the data acquisition and data processing.

2.9. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Scavenging Activity

This antioxidant assay was conducted as formerly performed by Lee et al. [18] with minor modifications. A sample concentration of 100 μg/mL was prepared. A total volume of 150 μL consisting of 50 μL of samples and 100 μL 0.059 mg/mL DPPH solution were transferred into each well and mixed well. The mixture was subjected to 30 min incubation in the dark condition and the absorbance was read at 525 nm on a microtiter plate spectrophotometer. In this assay, quercetin was used as positive control. The scavenging activity (SA) was calculated using the following formula:

$$\text{SA \%} = [(A_o − A_s)/A_o] \times 100\% \tag{2}$$

whereby A_o and A_s are the absorbance values of the reagent blank and samples, respectively. The experiment was repeated three times for each sample and the values were stated as percentage inhibition (%).

2.10. Statistical Analysis

The statistical analysis and Pearson's correlation test of the yield, TPC, TFC, TLC, TAC, TFCGC and DPPH free radical scavenging activity assays were performed via the Minitab and GraphPad statistical software. The values were presented as the mean ± standard deviation of three replications. Values labelled with different letters were significantly ($p < 0.05$) different. To evaluate the significant difference of each parameter, a one-way analysis of variance (ANOVA) completed by Tukey's test was employed.

3. Results and Discussion

3.1. Ultrasound-Assisted Extraction

3.1.1. The Effects of Solvent Extraction

The selection of extraction solvents is vital for extracting compounds from plants as it will govern the type and quantity of extracted phytochemical constituents. Figure 2A shows the yields of OPL crude extracts obtained using different extraction solvents. The different extraction solvents expressed different efficiencies for the extraction of phytoconstituents from OPLs. Methanol and its mixture with other solvents displayed a higher capability for extraction, compared to ethyl acetate and hexane. Among the different methanol-containing solvents, absolute methanol showed the highest yield with a percentage yield of 24.16%, followed by 4:1 methanol–water and 1:1 methanol–ethyl acetate with percentage yields of 23.99 and 19.17%, respectively. However, there was no significant difference between the yields of absolute methanol and 4:1 methanol–water OPL crude extracts. These results revealed that most of the phytochemical constituents in OPLs were hydrophilic in nature as the constituents were readily solubilized in polar solvents in comparison with non-polar solvents. These findings were consistent with former reports, in which the highest extraction yield was obtained using aqueous solvents [21,22].
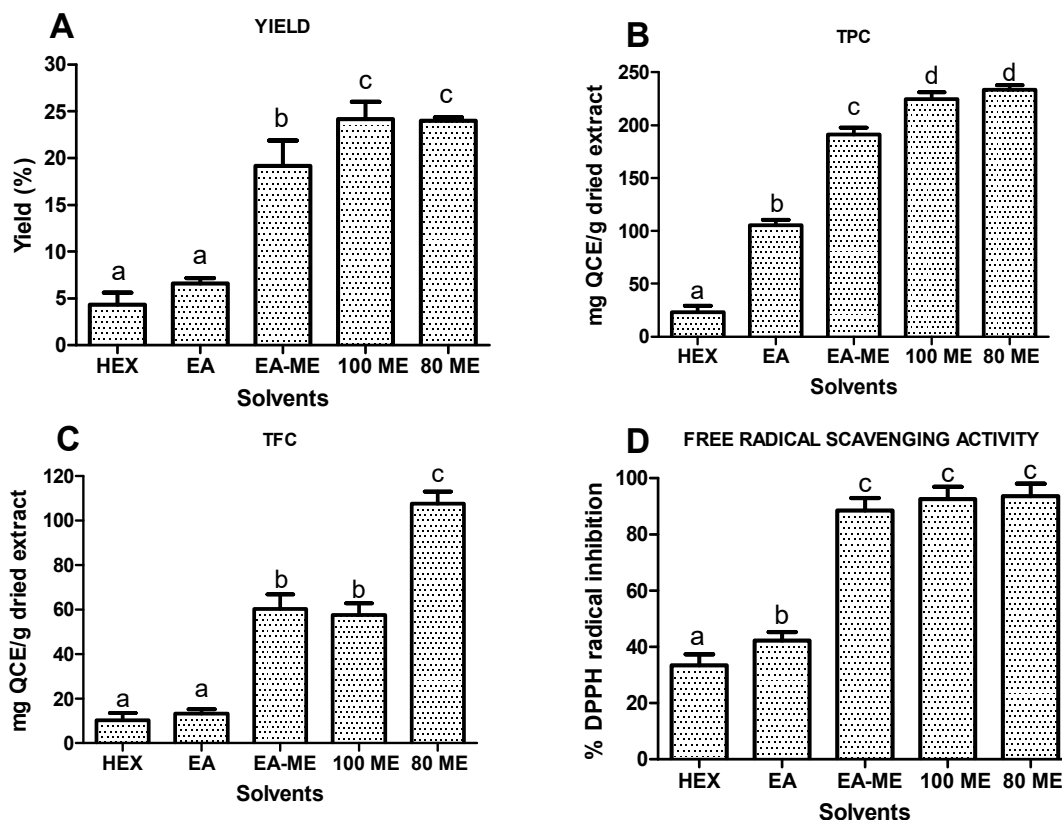
Figure 2. The effect of solvent on the extraction % yield (**A**), total phenolic content (TPC) (**B**), total flavonoid content (TFC) (**C**), and DPPH free radical scavenging activity (**D**) from OPLs. HEX, hexane; EA, ethyl acetate; EA–ME, 1:1 ethyl acetate–methanol; 100 ME, absolute methanol; 80 ME, 4:1 methanol–water. Different letters indicate significant difference between tested parameters ($p < 0.05$).

The TPCs of OPLs in association with the different extraction solvents are shown in Figure 2B. The results of TPC showed similar results as in percentage yield, whereby methanol and its mixture

with other solvents showed better efficiency in the extraction of phenolics from OPLs. Among the extracts prepared by mixtures of methanol with other solvents, the 4:1 methanol:water extract possessed the highest TPC at 233.47 mg QCE/g, followed by absolute methanol and 1:1 methanol–ethyl acetate extracts with TPC values of 224.61 and 191.05 mg QCE/g, respectively. The quantity of phenolic compounds being extracted was significantly affected by the mixtures of solvents due to its greater capability to disintegrate the constituent matrix and to liberate the congregated phenolics [23,24]. Hence, this explains the reason why there was a greater phenolic extractability in certain mixtures, such as aqueous methanol compared with absolute methanol [25].

Furthermore, a TFC assay was carried out to quantify the flavonoids in OPLs as shown in Figure 2C. The results revealed that TFC also follows a similar trend as with percentage yield and TPC. The 4:1 methanol water was again the most efficient extracting solvents with a TFC value of 107.66 mg QCE/g, followed by 1:1 methanol–ethyl acetate, absolute methanol, ethyl acetate, and hexane with TFC values of 60.44, 57.58, 13.32, and 10.34 mg QCE/g, respectively. Supported by the principle of "like dissolves like", these results reveal that most of the flavonoids present in the OPLs exhibited high polarity. However, some flavonoids could also be extracted by non-polar and moderate polar solvents. Hence, we anticipated that OPLs would consist of a diverse range of phenolics, including flavonoids with different polarities.

The antioxidant activities of OPL extracts acquired from various solvents are presented in Figure 2D. The results were stated as the percentage inhibition (%) of OPL extracts at the concentration of 100 μg/mL. Solvents with different polarities portrayed significantly different DPPH free radical scavenging activity, except for absolute and aqueous methanol. In accordance with the highest TPC and TFC, the 4:1 methanol–water extract exhibited the highest antioxidant activity with a percentage inhibition of 93.65%, followed by absolute methanol, 1:1 methanol–ethyl, acetate ethyl acetate, and hexane extracts, with their percentages of inhibition at 92.57, 88.58, 42.26, and 33.36%, respectively. The results suggest the efficiency of aqueous solvents in extracting antioxidant compounds, which is in agreement with previous reports [21,26]. The addition of water to the solvent, even at low quantities, was capable of enhancing its antioxidant extracting capability [24,27].

3.1.2. The Effects of Solid–Liquid Ratio Extraction

The connections between the yields of crude extracts of OPLs and the different solid–liquid ratios are demonstrated in Figure 3A. The trend shows that the percentage yield increased from 1:20 to 1:50 with the values of 22.10 and 24.60%, respectively, before the yield started to fluctuate. It was surmised that at 1:50, the percentage yield achieved its optimum level. This research has shown that the solid–liquid ratio within the tested range has no significant effect on the percentage yield. Contrary to the mass transfer rules, the driving force during mass transfer in the concentration gradient between the solid and liquid is larger when a higher ratio is applied [28]. The increase in the solvent volume did not produce a significant increase in percentage yields when it has already reached its maximum level [28].

The relationship between the TPC and TFC of OPLs with solid–liquid ratios are expressed in Figure 3B,C, respectively. Generally, the phenolic content increases when the solid–liquid ratio increases from 1:20 to 1:50, with TPC values of 178.90 and 207.63 mg QCE/g, respectively, and subsequently decreases gradually until the ratio of 1:70, with a TPC value of 192.88 mg QCE/g. TFC followed a similar trend as TPC. The total flavonoid content obtained from OPLs ranged between 33.61 and 46.15 mg QCE/g. The higher and lower TFC values were attained at 1:50 and 1:20 solid–liquid ratios, respectively. When the OPL powder and solvent ratios increased from 1:20 to 1:50, the TFC values also increased, and subsequently started to decrease from 1:50 to 1:70. According to Jun [29], the dissolution of bioactive components into the solvent was a physical process. The trend was classified into two parts. In the first part where the ratio ranges between 1:20 and 1:50, as the volume of the solvent increases, the chances of bioactive components releasing and contacting with the solvent increases, generating greater leaching-out rates. In the second part where the ratio ranges between 1:50 and 1:70, the decrease

in TPC and TFC values at a higher solid–liquid ratio was suggested to be due to the reduction in ultrasound energy dispersion and the accumulation of impurities in the solvent, which further hindered the liberation of constituents, including flavonoids into the solvent [30]. The solid–liquid ratio of 1:50 was the optimum ratio for phenolic and flavonoid extraction from OPL extracts.
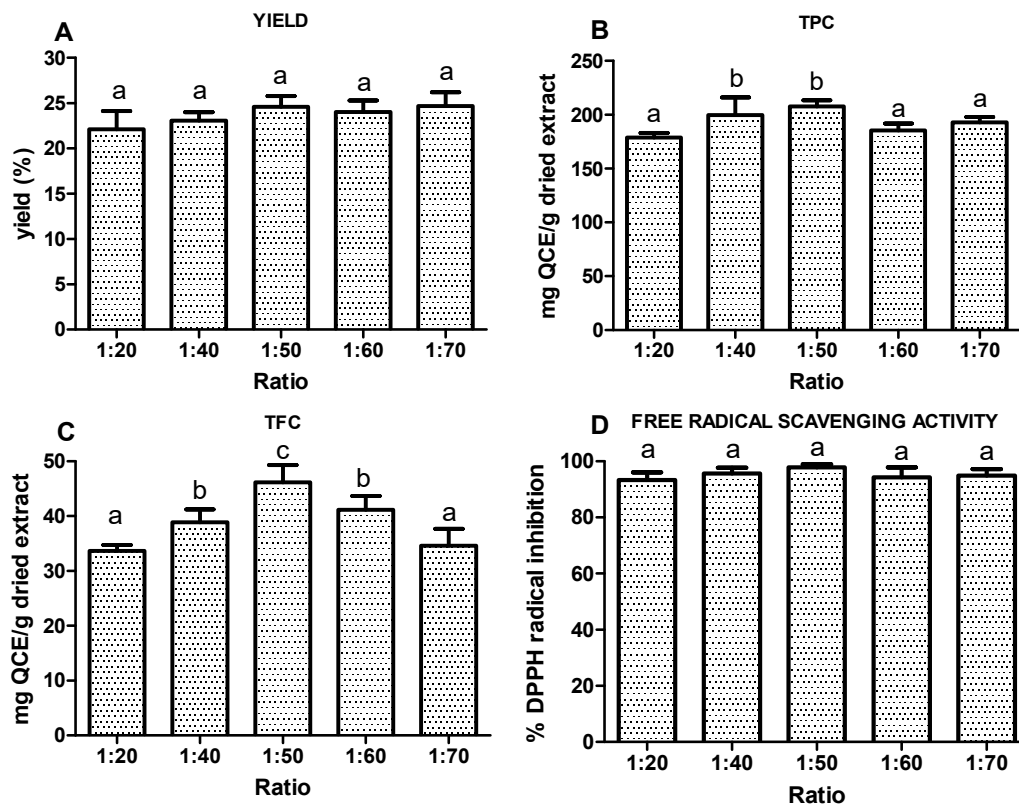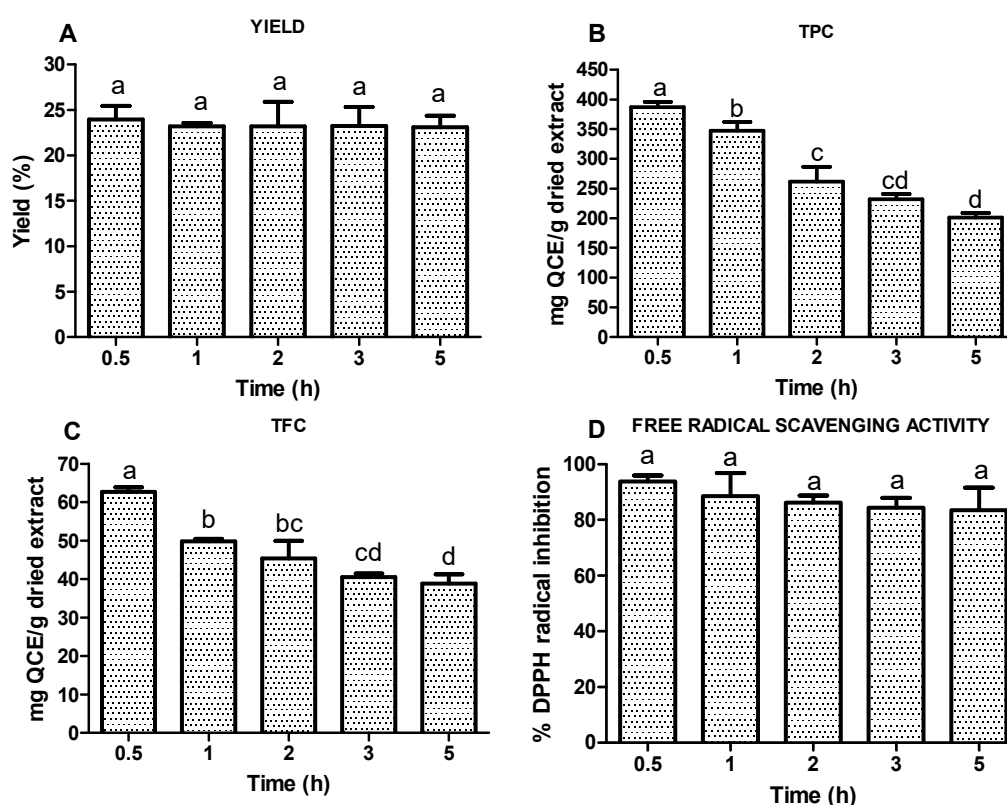

Figure 3. Effect of solid-to-liquid ratio on the extraction % yield (**A**), TPC (**B**), TFC (**C**), and DPPH free radical scavenging activity (**D**) from OPLs. Different letters indicate significant difference between tested parameters ($p < 0.05$).

The antioxidant activities of OPL extracts gained from different solid–liquid ratios are illustrated in Figure 3D. Statistically, there was no significant difference between the percentage inhibitions of DPPH free radical scavenging activity within the tested solid–liquid ratio. The optimum volume of solvents was regarded as its ability to cover the OPL powder appropriately apart from its operating costs, solvent consumption, and the prevention of saturation effects [31]. Though the solid–liquid ratio did not have a great significant effect on the percentage yield and DPPH scavenging activity, the 1:50 ratio was observed to be relatively better than the other tested solid–liquid ratios, especially for TPC and TFC. A similar ratio was applied in previous studies using the same plant materials [12,32,33]. Hence, 1:50 was the optimum solid–liquid ratio employed in the extraction of phenolic constituents from OPLs.

3.1.3. The Effects of Extraction Time

Figure 4A illustrates the relationship between the yields of crude extracts of OPLs and the different contact times. The results show no significant difference in the percentage yield within the tested contact times. The percentage yield achieved its optimum level after 30 min. These situations could be associated to Fick's second law of diffusion, suggesting that a final equilibrium between the solute concentrations (OPL powder) and the bulk solution (solvent) could be achieved after a period of time, resulting in the decrement of the extraction yield [34].

Figure 4. Effect of contact time on the extraction % yield (**A**), TPC (**B**), TFC (**C**), and DPPH free radical scavenging activity (**D**) from OPLs. Different letters indicate significant difference between tested parameters ($p < 0.05$).

The effects of the different contact times on the TPC and TFC of OPLs are demonstrated in Figure 4B,C, respectively. Both the TPC and TFC values decreased with the increase in contact times between the OPL powder and the solvent. The highest TPC and TFC were at 0.5 h, with their respective values at 387.35 and 62.66 mg QCE/g. The prolonged extraction led to a reduction in phenolic content in the crude extracts. This might be due to compound oxidation as a result of the prolonged exposure to environmental influences such as temperature, light, and oxygen [35,36].

The antioxidant activity of OPL extracts observed in different contact times are presented in Figure 4D. Similar to the results of TPC and TFC, the antioxidant activity decreased from 93.76 to 83.50% as the contact times increased from 0.5 to 5 h. The result suggests that the highest antioxidant activity was likely contributed by the phenolic compounds present in OPL extracts at 0.5 h. However, the different contact times generated a non-significantly different DPPH free radical scavenging activity. Hence, a prolonged extraction time was deemed unnecessary to extract more phenolic compounds from OPLs. The delayed extraction may cause an excessive solvent loss via vaporization, which directly reduces the volume of extraction solvent and subsequently decreases the solid–liquid ratio [37]. Furthermore, the increased extraction duration is time consuming and uneconomical from a business perspective. In consideration of the yield of phenolic compounds from the OPLs, 0.5 h could be considered as the optimum contact time for extraction. A similar result was demonstrated when an optimization of contact times on phenolic compounds from grapes *(Vitis vinifera)* was carried out [25].

3.1.4. The Effects of Extraction Temperature

In the last step of this optimization study, the selection of suitable extraction temperature was performed. The yields of the OPL crude extracts in relation to the various temperatures are portrayed in Figure 5A. The results suggest that changing extraction temperatures expresses a non-significantly

different extraction yield. The percentage yield achieved its optimum level at 25 °C. The effects of applying different temperatures on the TPC and TFC of OPLs are illustrated in Figure 5B,C, respectively. Both the TPC and TFC decreased with the increase in temperature up to 60 °C. The highest TPC and TFC were at 25 °C, with values of 335.30 and 60.67 mg QCE/g, respectively. The continuous raising of extraction temperature could cause a possible concurrent degradation of constituents that were already released at lower temperature [38]. The denaturation of membranes and a possible decomposition of constituents caused by hydrolysis, polymerizations, and internal redox reaction may occur and influence the quantification of phytochemical constituents [39]. Flavonoids is an example of a phenolic compounds that is heat sensitive, hence the elevation of temperature may potentially degrade such thermo-sensitive phenolic compounds. Several studies have previously reported that high temperatures degraded phenolic compounds such as total flavanone glycoside in peach fruit [40], proanthocyanidins in grape [41], and anthocyanins in black current [42]. In addition, high temperatures could lead to the formation of new compounds that are absorbed at the same wavelength as phenolic compounds are [41]. This may explain the sudden increase in TPC and TFC at 70 °C and 60 °C, respectively.
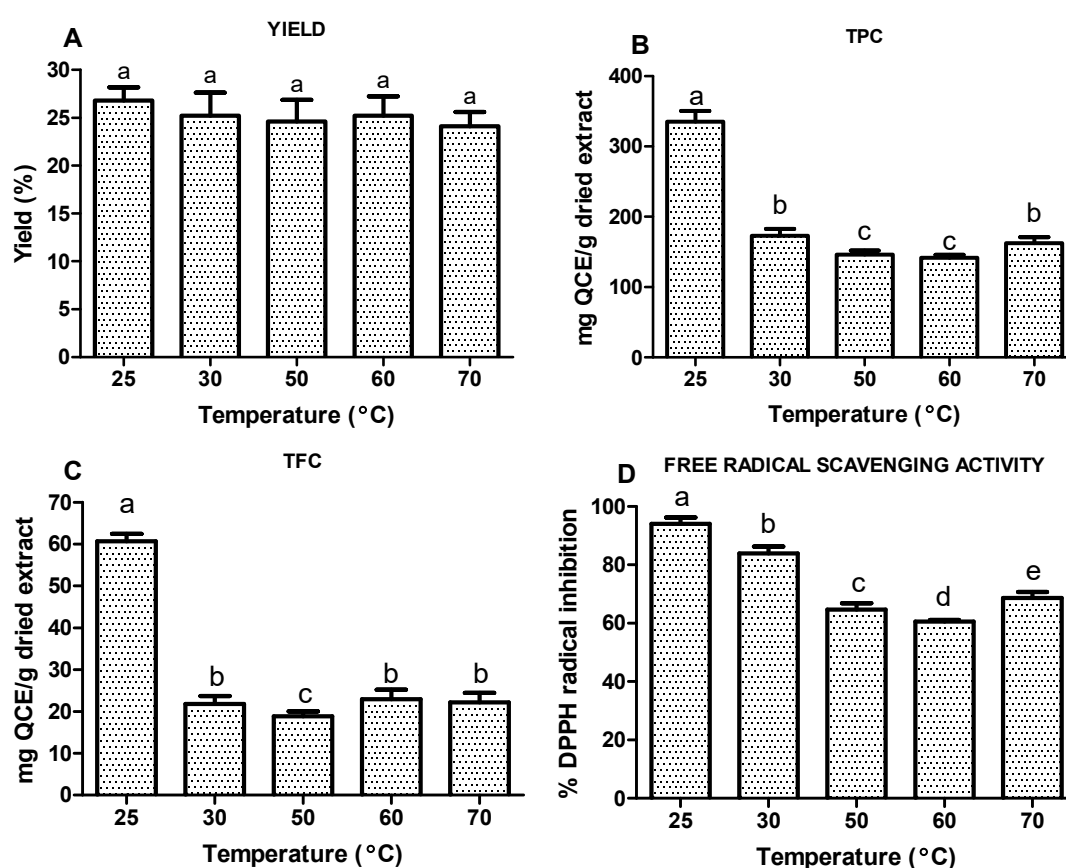
Figure 5. Effect of temperature on the extraction % yield (**A**), TPC (**B**), TFC (**C**), and DPPH free radical scavenging activity (**D**) from OPLs. Different letters indicate a significant difference between the tested parameters ($p < 0.05$).

In line with the trend shown in TPC and TFC, the antioxidant activity decreased as the temperature was raised from 25 to 60 °C, with the percentages of inhibition at 94.06 and 60.54%, respectively, and then a sudden increase at 70 °C with the percentage inhibition at 68.62%, as indicated in Figure 5D. The obtained results suggest that the high antioxidant activity was contributed by the phenolic compounds present in OPL extracts at 25 °C. The different temperatures conveyed a significantly different antioxidant activity. The extraction could be carried out without imposing heat on the OPL

sample, as it could yield the optimum amount of phenolic compounds that had considerable influence on antioxidant activity at room temperature. From an industrial-scale viewpoint, this could indirectly reduce operational costs. Similar results were described when the optimization of temperature on the extraction of phenolic constituents from various plant materials such as grapes [25] and peach fruits [40] were performed.

3.1.5. Pearson Correlation Analysis

The correlations between the TPC, TFC, and antioxidant activity under various UAE conditions were evaluated to investigate the relationships between antioxidant capacities and phenolic compounds present in the OPL extracts. Pearson's coefficient of each parameter is tabulated in Table 1. Theoretically, the larger the absolute value of the coefficient, the stronger the relationships. The findings revealed that the TPC and antioxidant activity was strongly and positively correlated, with $r = 0.965, 0.798$, and 0.858 for the solvent, solid–liquid ratio, and temperature, respectively. As for the time parameter, the TPC and antioxidant activity were moderately correlated with $r = 0.596$. The higher the TPC in the OPL extracts, the higher the percentage inhibition of DPPH free radicals. A similar trend was observed for the TFC with antioxidant activity. The TFC of solid–liquid ratio, temperature, and solvent, with $r = 0.775$, 0.779, and 0.640, respectively, were considered as parameters that possess strong correlations with the antioxidant activity, while for time, the TFC and antioxidant activity only showed moderate correlation with $r = 0.585$. Moreover, very strong positive correlations between TPC and TFC for time and temperature were observed, with $r = 0.930$ and $r = 0.983$, respectively, while a strong positive correlation between the TPC and TFC for a solvent with $r = 0.696$ and moderate correlations for a solid–liquid ratio with $r = 0.538$ were also observed. Flavonoids are a subfamily of phenolic compounds. Hence, the higher the flavonoid content in the extracts, the higher the amount of phenolic compounds, resulting in the higher antioxidant activity of these extracts. Additionally, the synergism of phenolics with one another may contribute to the overall observed antioxidant capacity. Similar results were also reported by Mokrani and Madani [40].

Table 1. Pearson's coefficient of polyphenolic content and antioxidant activity at different ultrasound-assisted extraction conditions.

| Pearson's Coefficient (*r*)		TPC			TFC		
Solvent	DPPH	0.965 *			0.640 *		
	TFC	0.696 *					
Ratio	DPPH		0.798 *			0.775 *	
	TFC		0.538 *				
Time	DPPH			0.596 *			0.585 *
	TFC			0.930 *			
Temperature	DPPH				0.858 *			0.779 *
	TFC				0.983 *		

Values with asterisk (*) were significant at $\alpha = 0.05$ based on Pearson's correlation coefficient method.

3.2. Effects of Acid Hydrolysis Conditions on Quantitative Determination of Flavonoid C-Glycosides in OPLs

3.2.1. Identification of Flavonoid *C*-Glycosides Using LC–MS/MS and UHPLC–UV/PDA Analysis

Figure 6 and Table 2 highlight the flavonoid *C*-glycosides that were putatively identified after acid hydrolysis. The identification of the compounds was performed by referring to an online database and previous works [12,13,43,44]. After confirmation with commercial standards, peaks 3, 4, 5, and 6 were assigned to isoorientin, orientin, vitexin, and isovitexin, respectively. In total, at least six flavonoid *C*-glycosides comprising of three luteolin (luteolin-6,8-di-*C*-hexose (1), luteolin-6-*C*-hexose (3), and luteolin-8-*C*-hexose (4)) and three apigenin derivatives (apigenin-6,8-di-*C*-hexose (2), apigenin-8-*C*-hexose (5), and apigenin-6-*C*-hexose (6)) were found after the acid treatment. These six flavonoid *C*-glycosides were quantified relatively, whereby the total apigenin content (TAC) established resulted from the addition of compounds 2, 5, and 6, which were equivalents to vitexin. Meanwhile,

the total luteolin content (TLC) was determined as a sum of compounds 1, 3, and 4, which were equivalents to orientin. To obtain total flavonoid *C*-glycoside content (TFCGC), the total amount of TAC and TLC was added. This approach was applied to relatively quantify the amount of total apigenin, total luteolin, and total flavonoid *C*-glycosides recovered from acid hydrolysis at different conditions, including acid molarity, incubation time, and incubation temperature.
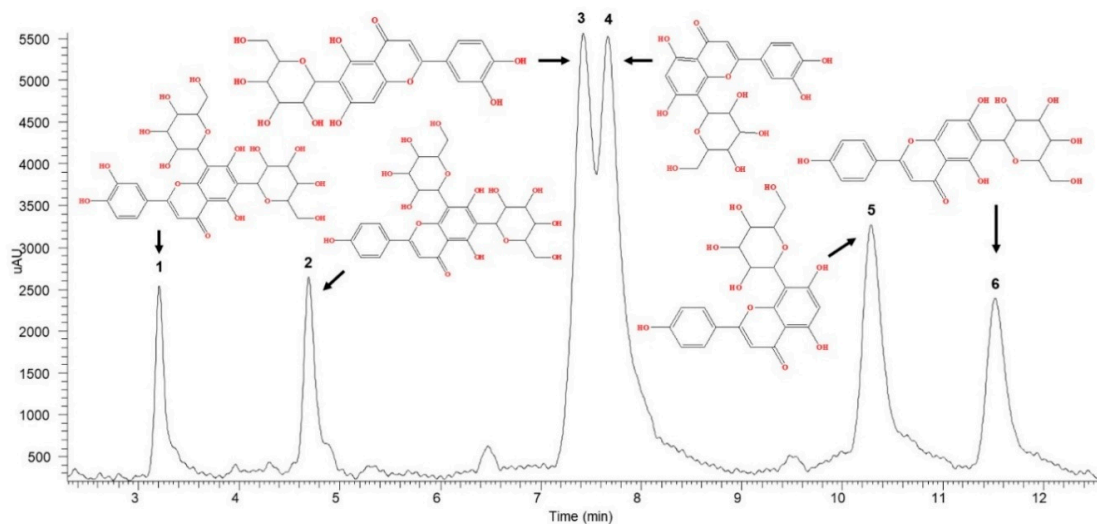
Figure 6. UHPLC–UV/PDA chromatogram of acid hydrolysed OPL extract at 340 nm with assigned chemical structures. Peak assignment: 1, luteolin-6,8-di-*C*-hexose; 2, apigenin-6,8-di-*C*-hexose; 3, isoorientin; 4, orientin; 5, vitexin and 6, isovitexin. Peaks 3–6 were confirmed by commercial standards. uAU stands for micro-Absorbance Units.

Table 2. Identification of flavonoid *C*-glycosides after acid hydrolysis by UHPLC–UV/PDA and LC–MS/MS.

Peak	t_R (min)	λ_{max}, (nm)	[M-H]$^-$ (m/z)	Formula	Key MS/MS Fragments (m/z)	Proposed Compound
1	3.20	272, 348	609.1411	$C_{27}H_{30}O_{16}$	519.1104 489.0998, 429.0786, 399.0696, 369.0585	Luteolin-6,8-di-*C*-hexose
2	4.70	272, 336	593.1464	$C_{27}H_{30}O_{15}$	503.1155, 473.1051, 383.0739, 353.0638	Apigenin-6,8-di-*C*-hexose
3	7.40	270, 348	447.0896	$C_{21}H_{20}O_{11}$	357.0588, 339.0480, 327.0483, 297.0379, 285.0381	Isoorientin (Luteolin-6-*C*-hexose)
4	7.80	270, 350	447.0896	$C_{21}H_{20}O_{11}$	357.0587, 339.0476, 327.0485, 297.0378, 285.0380	Orientin (Luteolin-8-*C*-hexose)
5	10.40	270, 338	431.0947	$C_{21}H_{20}O_{10}$	341.0639, 323.0529, 311.0536, 283.0589	Vitexin (Apigenin-8-*C*-hexose)
6	11.60	270, 338	431.0947	$C_{21}H_{20}O_{10}$	341.0638, 323.0536, 311.0536, 283.0588	Isovitexin (Apigenin-6-*C*-hexose)

3.2.2. The Effects of Acid Molarity

Acid hydrolysis at different acid molarities affects the total phenolic content (TPC), total flavonoid content (TFC), total apigenin content (TAC), total luteolin content (TLC), total flavonoid *C*-glycosides (TFCGC), and DPPH free radical scavenging activity of OPL extracts as presented in Figure 7. Figure 7A highlights the total phenolic content (TPC) after treating with acid at different concentrations. The results show that TPC decreased gradually as the acid molarity increased from 0.2 M to 12 M with TPC values ranging between 60.94 and 140.04 mg GAE/g. Previous studies reported that OPLs without

acid treatment contained organic acids, phenolic acid glycosides, and flavonoid glycosides [12,13,43,44]. However, it was highly likely that these compounds degraded at higher acid molarities due to exposure to harsh conditions, resulting in low TPC values. Our results were similar with the findings reported by previous works [44–47]. Moreover, the acid hydrolysis resulted in various total flavonoid content (TFC) values when crude OPL extracts were treated with different acid concentrations. An interesting trend was observed in the TFC experiment, whereby the extracts gave two optimum acid concentrations with the highest TFC values at two concentrations: 1 M and 6 M, their respective TFC values 31.57 and 33.98 mg QCE/g. The results suggest that some flavonoids present in OPLs are resistant to acid at both mild and harsh conditions. These findings were supported by previous works that also studied the acid hydrolysis of flavonoids [46,48].
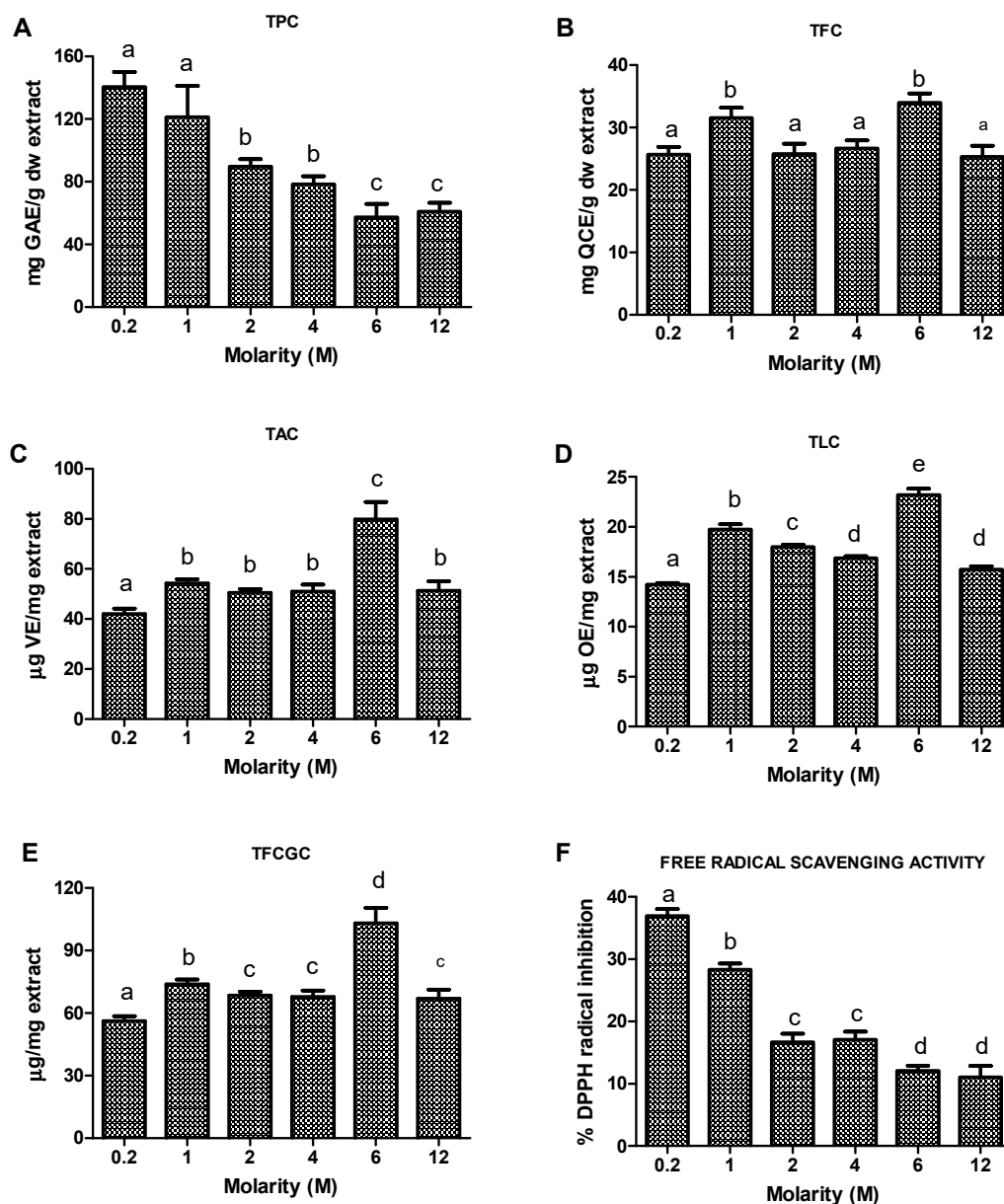
Figure 7. Effects of acid molarity on the acid hydrolysis for TPC (**A**), TFC (**B**), total apigenin content (TAC) (**C**), total luteolin content (TLC) (**D**), total flavonoid *C*-glycoside content (TFCGC) (**E**), and DPPH free radical scavenging activity (**F**). Different letters indicate significant difference between tested parameters ($p < 0.05$). DW stands for dried weight.

The total apigenin content (TAC) was obtained based on the addition of putatively identified peaks that belonged to apigenin derivatives and were closely referred to in previously published works [12,13]. The highest TAC was produced at 6 M, with a TAC value of 79.92 μg vitexin equivalent (VE)/mg. The findings suggest that the optimum TFC values at 6 M was mainly contributed by apigenin derivatives, including apigenin-6,8-di-*C*-hexose, vitexin, and isovitexin, which were present in the OPL extract. The total luteolin content (TLC) was found based on the sum of the putatively named peaks that belonged to luteolin derivatives and was closely referred to in previously published work [12,13]. The relationships between the TLC with different acid concentrations are exhibited in Figure 7D. TLC was found to be highest at 6 M, with a TLC value of 23.79 mg orientin equivalent (OE)/g, followed by 1 M with a TLC value of 19.76 μg OE/mg. Luteolin derivatives including luteolin-6,8-di-*C*-hexose, orientin, and isoorientin, are among the flavonoid *C*-glycosides present in the OPL extracts that can withstand harsh conditions. Hence, the TLC results complemented the trends shown by TFC, whereby 1 or 6 M acid concentration was suggested to be optimum conditions for acid hydrolysis. As a result of the accumulation of recognized peaks that belonged to apigenin and luteolin derivatives, the total flavonoid *C*-glycoside content (TFCGC) was established. The relationship between the TFCGC with different acid concentrations are indicated in Figure 7E. The sum of the TAC and TLC resulted in the highest TFCGC value of 103.11 μg/mg when the crude OPL extract was treated with acid at 6 M. Acid hydrolysis was previously utilized to obtain TFCGC, including isoorientin, orientin, vitexin, isovitexin, vicenin-2, an lucenin-2 [12,49,50].

The antioxidant activity of acid hydrolysed OPL extracts at different acid concentrations is presented in Figure 7F. Similar to the results of TPC, the free radical scavenging activity decreased as acid molarities increased from 0.2 to 12 M, with their range of percentage inhibition at 36.91 to 11.03%. The increment of acid molarity decreases the antioxidant activity due to the elimination of an abundance of antioxidant compounds present in OPLs that are known to possess high free radical scavenging activity. Some of the compounds that cannot withstand high acid concentrations include sugars, organic acids, phenolic acids, phenolic acid glycosides, and catechins [12,51]. For the subsequent experiments, 6 M was selected as an optimum acid molarity for the hydrolysis of flavonoid glycosides present in OPLs.

3.2.3. The Effects of Incubation Time

Figure 8 shows the TPC, TFC, TAC, TLC, TFCGC, and antioxidant activity of OPL extracts after acid hydrolysis at different incubation times. The crude OPL extracts contain phenolic compounds that were hydrolysed at different incubation times. The results shown in Figure 8A suggest that as the incubation time increased from 10 to 45 min, the TPC values increased from 99.69 to 123.99 mg GAE/g and remained constant until 90 min incubation. The lowest TPC was obtained at 120 min incubation period with a TPC value of 77.40 mg GAE/g. Our recent study suggests that OPLs contain arrays of phenolic compounds, including phenolic acid glycosides, apigenin derivatives, luteolin derivatives, and catechin derivatives [13]. However, after the acid treatment at different incubation times, there was a high possibility that these compounds, especially phenolic acid glycosides, were reduced due to the prolonged incubation period, resulting in low TPC values. Different TFC values were obtained when crude OPL extracts were incubated at different incubation times, as shown in Figure 8B. The incubation period for acid hydrolysis showed that the 90 min incubation resulted in the highest TFC value at 36.69 mg QCE/g. However, starting from 30 to 90 min, the TFC values were observed to reach their optimum levels, reflected by the high TFC values with persistent fluctuations. A prolonged incubation time may lead to the degradation of flavonoids, as observed at 120 min when the TFC value was lowered to 23.97 mg QCE/g.
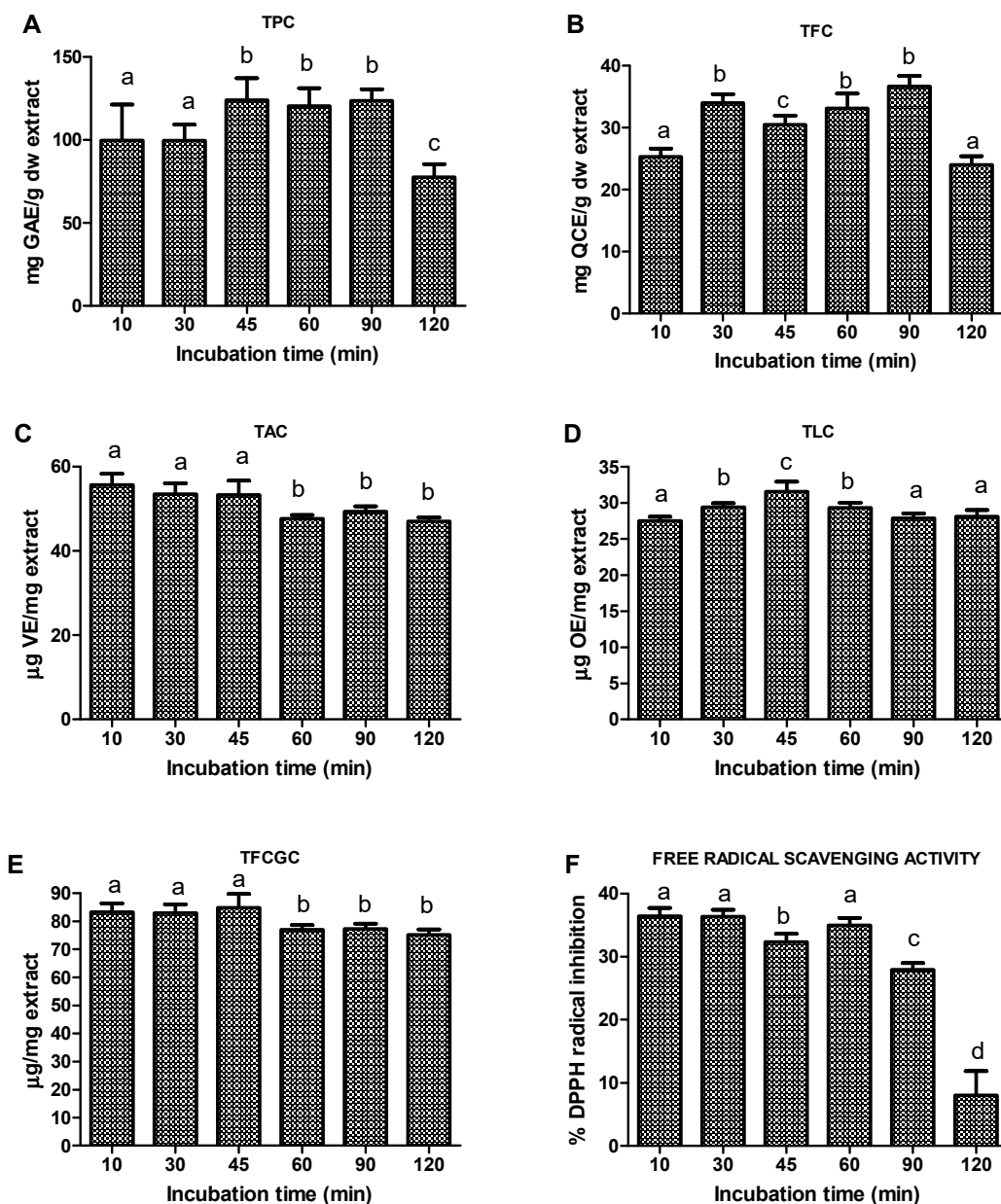
Figure 8. Effects of incubation time on the acid hydrolysis for TPC (**A**), TFC (**B**), TAC (**C**), TLC (**D**), TFCGC (**E**), and DPPH free radical scavenging activity (**F**) from OPLs. Different letters indicate significant difference between tested parameters ($p < 0.05$). DW stands for dried weight.

The influences of various incubation times on TAC and TLC are shown in Figure 8. Figure 8C,D, respectively. The incubation times showed that the TAC values reduced gradually as the time increased. There was a significant decrease in TAC values at the 45 and 60 min incubation periods, with their respective values of 53.26 and 47.60 µg VE/mg, indicating that the apigenin derivative compounds degraded at prolonged incubation times. Moreover, TLC reached the highest value at 45 min incubation time, with 31.56 µg OE/mg. Total flavonoid *C*-glycoside content (TFCGC) showed insignificant change during the 10–45 min incubation period. However, in between the 45 and 60 min incubation times, there was a significant decrease in TFCGC values from 84.82 to 76.94 µg/mg. Hence, TFCGC could be obtained optimally when the crude OPL extract was treated with acid during a 45 min incubation period.

The antioxidant activity of acid hydrolysed OPL extracts at different incubation times is presented in Figure 8F. A non-significantly different DPPH free radical inhibition from 10 to 30 min incubation

time was obtained with a percentage inhibition of 36.41%. Furthermore, the inhibition started to decrease as the incubation period was prolonged. Acid hydrolysis in general decreases the antioxidant activity compared with crude OPL extracts. This is due to the degradation of the abundance of antioxidant constituents such as phenolic acids present in crude OPL extracts, which are known to exhibit high free radical scavenging activity at prolonged incubation times [45]. For the following experiments, the optimum incubation time for the hydrolysis of flavonoid glycosides present in OPLs was set at 45 min.

3.2.4. The Effects of Incubation Temperature

The TPC, TFC, TAC, TLC, TFCGC, and antioxidant activity of OPL extracts after acid hydrolysis at different incubation temperatures are presented in Figure 9. Figure 9A represents the total phenolic content (TPC) after treating with acid at different incubation temperatures. The results show a decrease in TPC with the increment in incubation temperatures, with a TPC value of 134.24 mg GAE/g at 65 °C and decreased to 68.80 mg GAE/g at 90 °C. Interestingly, at 95 °C, the TPC value sharply increased to 126.33 mg GAE/g. Phenolic acid glycosides were previously reported to be present in crude OPL extracts, however, due to its thermosensitive properties, these compounds could not be found in hydrolysed OPL extracts, which were expected to be degraded when the extract was incubated at high temperatures [12,49]. Nonetheless, the unprecedented increase in the TPC value at 95 °C remains inconclusive until the TFC step was performed. The TFC values at different incubation temperatures starting from 65 to 95 °C showed a fluctuating pattern. The highest TFC value was obtained at 95 °C with a TFC value of 36.08 mg QCE/g. The presence of flavonoid *C*-glycosides including luteolin and apigenin derivatives in OPL extracts may explain the findings, whereby these compounds can withstand acid hydrolysis at high temperatures [12]. The results revealed that the optimum TFC values can be reached at high temperatures, preferably at 95 °C. The findings indirectly substantiate the sudden increase in the TPC value at 95 °C as previously discussed.

Figure 9C illustrates the effects of different incubation temperatures on the total apigenin content (TAC). Overall, there was no substantial difference between the TAC values at different incubation temperatures except at 65 and 95 °C, suggesting that apigenin derivatives can withstand high temperatures. Again, at 95 °C, the TAC was at its highest with a TAC value of 79.12 µg VE/mg. The total luteolin content (TLC) was insignificantly affected by the incubation temperatures in between 65 and 95 °C. TLC reached its highest value at 95 °C with a value of 20.97 µg OE/mg, a similar trend was reported by Salcedo et al. [49]. Accumulatively, the total flavonoid *C*-glycoside content (TFCGC) was not influenced by incubation temperatures, indicated by the insignificant TFCGC at low (65 °C) and high temperatures (95 °C), with TFCGC values of 97.31 and 100.09 µg/mg, respectively. Overall, TFCGC could be obtained optimally when the crude OPL extract was incubated at 65 and 95 °C. The antioxidant activity of acid hydrolysed OPL extracts at different incubation temperatures are presented Figure 9F. A fluctuation trend was observed as the incubation temperature increased, with insignificant free radical scavenging inhibition in between 51.97 and 54.88% except at 90 °C, indicating that non-thermosensitive antioxidant compounds such as flavonoid glycosides including luteolin and apigenin derivatives could still resist various incubation temperatures [12,13,49]. The optimum incubation temperature was selected at 95 °C for the hydrolysis of flavonoid glycosides found in OPLs.
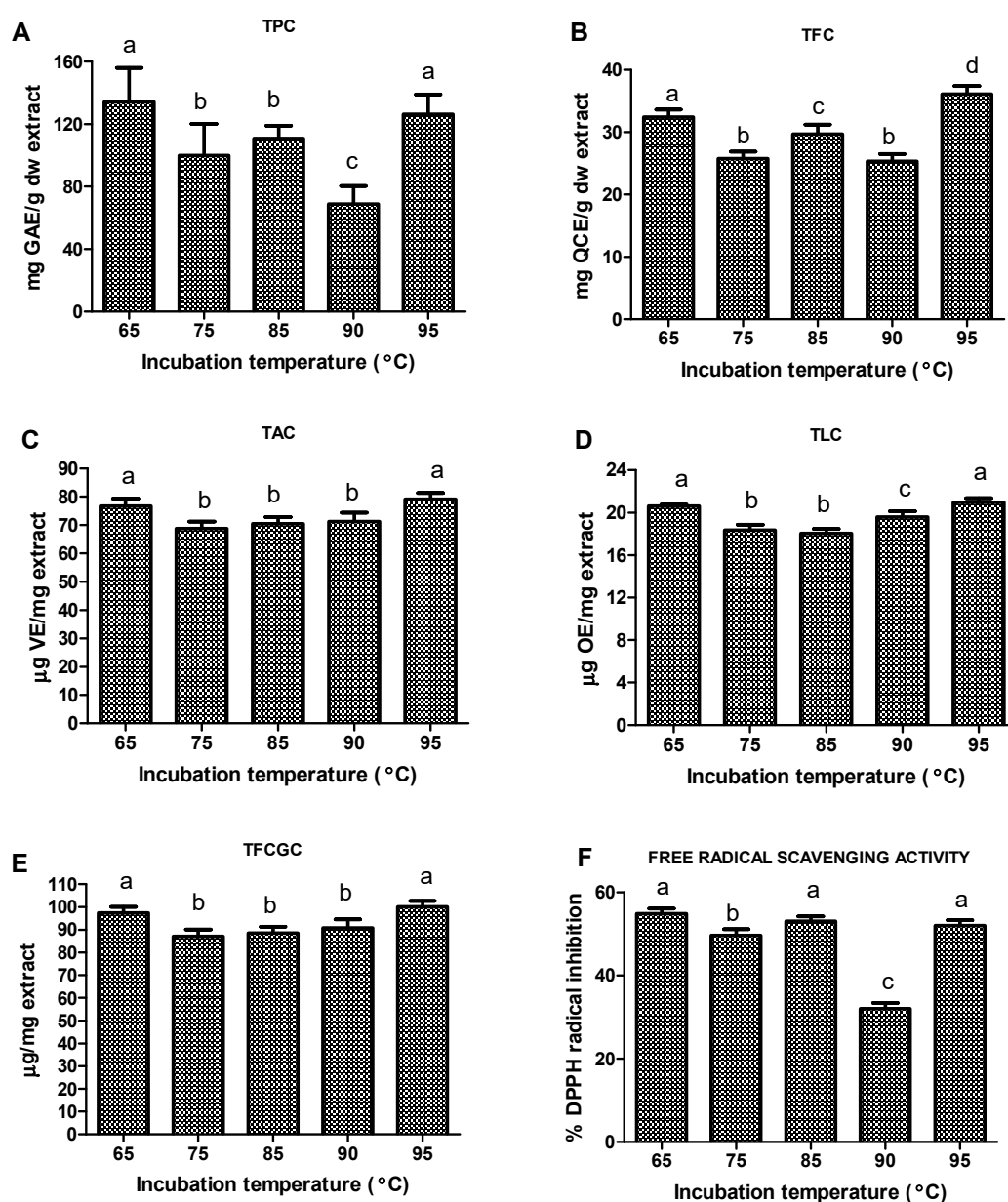
Figure 9. Effects of incubation temperature on acid hydrolysis for TPC (**A**), TFC (**B**), TAC (**C**), TLC (**D**), TFCGC (**E**), and DPPH free radical scavenging activity (**F**). Different letters indicate significant difference between tested parameters ($p < 0.05$). DW stands for dried weight.

3.2.5. Pearson Correlation Analysis

The relationships between TPC, TFC, TAC, TLC, TFCGC, and antioxidant activity under different acid hydrolysis conditions were investigated in order to examine the correlations between flavonoids, especially flavonoid *C*-glycosides, present in the acid hydrolysed OPL extracts. Pearson's coefficient of each parameter are tabulated as in Table 3. Again, the findings revealed that the TPC strongly correlated with antioxidant activity, with $r = 0.766$, 0.621, and 0.922 for acid molarity, incubation time, and temperature, respectively. The higher the TPC in acid hydrolysed OPL extracts, the higher the percentage inhibition of DPPH free radicals. Moreover, there was a strong correlation between the TFC and antioxidant activity for an incubation temperature with $r = 0.633$, while moderate correlations were observed for acid molarity and incubation time with $r = 0.479$ and 0.485, respectively. TAC was strongly correlated with DPPH free radical inhibition at different acid molarity and incubation time

with $r = 0.619$ and 0.643, respectively, while the TLC was moderately correlated with DPPH free radical inhibition for acid molarity with $r = 0.538$. The poor correlations of TAC, TLC, and TFCGC with antioxidant activity for incubation temperature suggest that the parameter within the tested range did not greatly influence the antioxidant activity. Overall, TFCGC hydrolysed at different acid molarity and incubated at different time periods showed a strong correlation with free radical inhibition with $r = 0.611$ and 0.664, respectively. Overall, the TFCGC hydrolysed at different acid molarity and incubated at different time periods showed a strong correlation with free radical inhibition with $r = 0.611$ and 0.664, respectively. The results suggest that flavonoid *C*-glycosides including luteolin and apigenin derivatives recovered from acid hydrolysis at optimized conditions were able to express their free radical scavenging activity. Tahir et al. also concluded that OPLs containing flavonoid *C*-glycosides showed good antioxidant activity [12].

Table 3. Pearson's coefficient of flavonoid *C*-glycosides and DPPH free radical scavenging activity at different acid hydrolysis conditions.

Pearson's Coefficient (r)		TPC	TFC	TAC	TLC	TFCGC
Acid molarity	DPPH	0.766	0.479	0.619	0.538	0.611
Incubation time	DPPH	0.621	0.485	0.643	0.280	0.664
Incubation temperature	DPPH	0.922 *	0.633	0.357	0.057	0.293

Values with an asterisk (*) were significant at $\alpha = 0.05$ based on Pearson's correlation coefficient method.

4. Conclusions

In the current study, the single factor experiment approach was applied to optimize the ultrasound-assisted extraction process of phenolic constituents from OPLs. Several variables were investigated, including the effects of solvent extraction, solid–liquid ratio, extraction time, and temperature. In general, the results demonstrated that the TPC, TFC, and antioxidant activity of the OPL extracts were significantly affected by the studied parameters. The optimal extraction conditions were selected based on the TPC, TFC, and antioxidant activity of the obtained crude extracts, as well as taking into consideration other factors such as its practicality, safety, and operational costs. Hence, it could be concluded that OPL powder could be extracted using 4:1 methanol–water at a 1:50 solid–liquid ratio, and sonicated for 0.5 h at 25 °C.

In a subsequent part of the study, the optimization of acid hydrolysis was conducted to obtain an optimum amount of flavonoid *C*-glycosides from OPLs by using a single factor experiment approach. Three main factors were considered in these experiments, including the effects of acid molarity, incubation time, and incubation temperature. In general, the results revealed that yield, TPC, TFC, TAC, TLC, TFCGC, and DPPH free radical scavenging activity were significantly affected by the investigated factors. It can be concluded that crude OPL extracts could be hydrolysed using 6 M hydrochloric acid and incubated at 95 °C for 45 min, resulting in OPL extracts containing optimal flavonoid *C*-glycosides. Since phenolic compounds such as flavonoid *C*-glycosides offer potential health benefits, significant attention should be made towards crude and acid hydrolysed OPL extracts and their applications in nutraceutical and pharmaceutical products. To the best of our knowledge, these studies are the first to report the influences of UAE parameters towards the recovery of phenolic compounds, and the effects of acid hydrolysis factors on the recovery of flavonoid *C*-glycosides from OPLs.

Author Contributions: K.S. and M.S.C.Z. conceptualized and designed the experiments; K.S. provided the research materials; M.S.C.Z., N.A.J. and J.X.Y. performed the experiments. M.S.C.Z. wrote the manuscript; K.S. and M.S.C.Z. analysed the data; K.S. and S.Y.L. validated the data and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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