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Chemical composition, antioxidant activity and extraction optimization of *Distichochlamys orlowii* rhizomes

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Abstract. *Distichochlamys orlowii* is an endemic ginger species in Viet Nam. The present study aimed to investigate the chemical composition, antioxidative capacity and extraction optimization of *D. orlowii* rhizomes (DO-R) for the first time. The results demonstrated that DO-R possessed more unsaturated fatty acids (53.10 %) than saturated fatty acids (46.90 %). Many unsaturated fatty acids of great health benefits were present, including trans-13-octadecenoic acid (C18:1), linoleic acid (C18:2), arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5). In addition, seven essential amino acids (EAA) were detected with 41.79 ± 1.02 mg. In comparison, ten non-essential amino acids (N-EAA) were found at a much higher amount of 252.09 ± 1.06 mg in 100 g DO-R. Valine and arginine were the most abundant EAA and N-EAA in DO-R. The methanol fraction from DO-R was shown to have a higher total phenolic content (TPC = 28.85 ± 0.74 mg GAE/g DW) and total flavonoid content (TFC = 14.03 ± 0.31 mg QE/g DW) than other fractions. In addition, this extract was also the most effective antioxidative agent with an IC_{50} value of 168.63 ± 4.83 and 153.80 ± 8.24 µg/mL against DPPH and ABTS radical, respectively. The highest extraction yield of DO-R in methanol (95.534 ± 0.593 mg/g DW) was achieved using response surface methodology with a Box-Behnken design under the optimal parameters: a solid-liquid ratio of 1:40 g/mL, an ultrasonic power level of 80 %, an extraction time of 60 min, and an extraction temperature of 58 °C. In conclusion, *D. orlowii* can serve as a source of high-potential compounds for further pharmacological applications.

Keywords: Distichochlamys orlowii, volatile components, fatty acids, amino acids, antioxidant capacity.

Classification numbers: 1.1.3, 1.2.1, 1.3.1.

1. INTRODUCTION

Ginger is one of the traditional spices with pungency and a unique aroma. Besides, this plant has been used as a folk medicine for hundreds of years with several bioactivities such as

antioxidant [1], antimicrobial [2], anti-inflammatory [3] or antitumor effect [4], etc. In Viet Nam, the endemic genus *Distichochlamys*, also known as black ginger, was discovered by Newman in 1995 [5]. Only four *Distichochlamys* species have been identified, including *D. citrea, D. orlowii, D. benenica,* and *D. rubrostriata* [6 - 8]. The *Distichochlamys* genus has been traditionally used by the Pako ethnic group as a medicine for colic, blood clotting, wound healing, and pus removal [9]. Recently, compounds isolated from *D. benenica* were shown to reduce the growth of several gram-positive bacteria and might have anti-inflammatory characteristics [9]. In the literature, *D. citrea* has attracted the most attention from scientists. A few studies on the chemical composition of this plant were previously investigated [10, 11]. In addition, several pharmacological effects of *D. citrea* were reported, such as antimicrobial [12, 13], antioxidant, and alpha-glucosidase inhibitory effect [10, 14]. *D. orlowii* (or black ginger Orlow) was found to distribute in a narrow area in a village in An Khe district, Gia Lai province, in 2001 [7]. To date, only one report on the essential oil composition of this species was published by Le *et al*. [15]. The study indicated that geranyl acetate (16.5 %), β-elemene (9.2 %), β-pinene (9.0 %), β-caryophyllene (7.9 %), α-humulene (4.9 %), and (Z)-citral (4.6 %) were the critical constituents in *D. orlowii*. These compounds have been documented to possess several pharmacological effects, such as antioxidative, anti-inflammatory, anti-viruses, and anti-tumor [16 - 19].

The present study aimed to evaluate the chemical composition (fatty acids, amino acids, total phenolic and flavonoid content) and the radical scavenging ability of the *D. orlowii* rhizomes. Moreover, the ultrasound-assisted extraction of DO-R was optimized to obtain the most significant extraction yield's extract which can serve as a source of bioactive compounds for further investigation.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Methanol, n-hexane, chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO), pentadecanoic acid (C15), saturated NaCl, and boron trifluoride (BF_3) were provided by Merck, Germany. Ninhydrin reagents and buffers were purchased from Biochrome, Ltd, UK. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid, trolox, and were obtained from Sigma- Aldrich.

2.1.2. Plant materials

Distichochlamys orlowii rhizomes (DO-R) were collected in Huong Minh commune, Vu Quang district, Ha Tinh province in 2021. Dr. Nguyen Quoc Binh, Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, identified this plant. A voucher specimen number SH1196 was stored at the Department of Life Sciences, University of Science and Technology of Hanoi.

2.2. Methods

2.2.1. Analysis of fatty acid content

Fatty acid extraction

300 mg of DO-R was extracted with 4 mL of chloroform/methanol (1:2) for one hour. Then, 30 µL of the C15 standard solution at 2.5 mg/mL was utilized as the internal standard. Next, chloroform (1.34 mL) and the saturated NaCl solution (1.34 mL) were put into the solution, shaking for 1 min after adding each substance. The sample was centrifuged for 5 minutes at 3000 rpm, and the lower layer containing the total fat was obtained.

Preparation of fatty acid ester derivatives

Dissolve the total fat in a 1 mL mixture of BF3-methanol (140 µL boron trifluoride and 860 µL methanol). Then, the solution was maintained for 1 hour at 80 °C. The mixture was shaken thoroughly every 5 min. After the solution reached room temperature, n-hexane (4 mL) was added, and carefully shake the solution for 15 seconds. Next, the upper layer was collected after centrifugation of the solution for 3 minutes at 1300 rpm. The supernatant was then aspirated and filtered before being transferred into a 2 mL glass vial for chromatographic analysis.

Analysis of fatty acids by GC-MS

One µL of the extracted fatty acids sample was separated by an Agilent HP-5MS UI capillary column (30 m × 0.25 mm × 0.25 µm, USA). The gas carrier was helium at a 1.4 mL/minute rate. A starting temperature of 100 °C was kept for 3 min before increasing to 220 °C at a 10 °C/minute rate. Then the temperature rose to 240 °C at a 4 °C/minute rate, then to 290 °C at a 20 °C/minute rate for 3 min. The total run time is 28 min.

2.2.2. Analysis of free amino acid content

100 mg of the DO-R was mixed with 3.75 mL of sodium loading buffer pH 2.2 for one minute. The sample was shaken for one hour at 1000 rpm**.** After filtration, the supernatant containing free amino acids (FAA) was collected. Then, 20 µL of the obtained FAA solution was introduced into an amino acid analyzer (Biochrom 30+, UK). The total run time is 60 min. A mixture of 23 standard amino acids (L-cystine, L-cysteic acid, L-valine, taurine, L-methionine, D, L-methionine sulfoxide, L-methionine sulfone, L-isoleucine, L-Leucine, L-aspartic acid, L-tyrosine, L-threonine, L-phenylalanyl, L-serine, L-histidine, L-glutamic acid, L-ornithine, L-proline, L-lysine, glycine, ammonia, L-alanine, and L-arginine) at 2.5 mM provided by Biochrom Ltd was used to calculate the FAA content by the formula:

$$FAA = C_{aa} \times M_{aa} \times V \times 10^{-3} \text{ (mg/100 g)}$$

where C_{aa} is the concentration of free amino acid (µmol/L), M_{aa} is the molecular mass of free amino acid (g/mol), and V is the volume of loading buffer (mL).

2.2.3. Analysis of total phenolic and total flavonoid content

3 g of DO-R were extracted at 40 ^{o}C for 1 hour using sonication with 200 mL of each solvent, including dichloromethane (DM), ethyl acetate (EA), methanol (ME), and ethanol (ET). The fractions were evaporated to obtain respective dried samples (DM: 0.051 g; EA: 0.13 g; ME: 0.23 g; ET: 0.15 g). The plant extracts were dissolved in DMSO in a concentration range from 100 to 1000 µg/mL.

Different plant extracts' total phenolic content (TPC) and total flavonoid content (TFC) were analyzed according to the previous method described by Le *et al.* [20]. The solution's absorbance was respectively recorded at 765 at 510 nm for TPC and TFC using a Microplate spectrophotometer (xMark, Bio-Rad). TPC was calculated as milligrams of gallic acid equivalent in one g of dried DO-R (mg GAE/g dried DO-R). TFC was determined as milligrams of quercetin equivalent in one g of dried DO-R (mg QE/g dried DO-R).

$$\text{TPC} = \frac{C_{GAE}}{C_0}, \text{TFC} = \frac{C_{QE}}{C_0}$$

where C_{GAE} is the content of gallic acid equivalent (mg), C_{QE} is the content of quercetin equivalent (mg), C_0 is the mass of the dried sample (g).

2.2.4. Antioxidant activity

The experiments followed the previous method with some modifications [21].

DPPH assay

200 µL of the sample composed of 10 µL plant extract and 190 µL DPPH (0.25 mM in methanol) was maintained at 40 °C for 10 minutes. The sample's absorbance was measured at 517 nm. Methanol and ascorbic acid were used as the negative and positive control, respectively. The inhibiting percentage against DPPH radical was determined using the equation:

$$\% \text{ I} = (1 - \text{ODs}/ \text{ODc}) \times 100 \%,$$

in which ODs is the sample's absorbance, and ODc is the negative control's absorbance.

ABTS assay

The $ABTS^{\cdot+}$ radical cation was generated after the potassium persulfate solution (2.45 mM in water) reacted with the ABTS solution (7 mM in water) at a ratio 1:1 (v/v) for 14 hours in the dark. 10 µL of the radical solution and 190 µL of the diluted solution of $ABTS^{\cdot+}$ (in ethanol) were incubated at 25 ^{o}C for exactly 10 minutes. A microplate reader measured the sample's absorbance at 734 nm. Trolox was utilized as the positive control in this experiment.

IC_{50} value was calculated as the sample's concentration for scavenging 50 % of the radicals used.

2.2.5. Optimization of DO-R's extraction yield

Single-factor experimental analysis

The single experimental design determined the impact of four factors, including ultrasonic power, solid-liquid ratio, extraction time, and extraction temperature on the DO-R's extraction yield. The experiment was constructed using various levels of every single factor to be evaluated while the other elements were kept at a constant level:

- The ultrasonic power's impact on the extraction yield was investigated at 30, 50, 60, 80, and 100 % with a solid-liquid ratio of 1:20 g/mL for 20 minutes and at 40 °C.

- The solid-liquid ratio's influence was evaluated at 1:10, 1:20, 1:30, 1:40, and 1:50 g/mL with 30 % ultrasonic power, 20 min extraction time, and 40 °C extraction temperature.

- The extraction time's effect was studied at 20, 30, 40, 60, and 90 minutes with 30 % ultrasonic power, 40 °C extraction temperature, and a 20 minutes extraction time.

- The extraction temperature's impact was determined from 30, 40, 50, 60 to 70 °C with 30 % ultrasonic power, 1:20 g/mL liquid-solid ratio, and a 20 minutes extraction time.

One gram of DO-R was extracted with methanol in specific conditions. Then, the supernatant was collected after filtration and centrifugation. Finally, the extraction yield of DO-R for each experiment was determined as milligrams of the dried extract in one gram of the dried weight sample (DW), following the formula:

$$Extraction\ yield\ \left(\frac{mg}{g}\right) = \frac{Mass\ of\ dried\ extract\ (mg)}{Mass\ of\ dried\ sample\ (g)}$$

Box-Behnken design (BBD)

The Box-Behnken design is an effective method commonly used in response surface methodology (RSM) to optimize the extraction of natural products [22]. Following single-factor tests, a BBD (Table 1) with four factors and three levels for each factor (-1, 0, +1) was used in this experiment to evaluate the impact of various factors on the DO-R's extraction yield simultaneously. A quadratic polynomial model describes the mathematical relationship between the independent variables and the response value.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + \sum_{i=1}^{4} \beta_{ii} X_i^2$$

in which the extraction yield (Y) is the predicted response, X_i and X_j are input variables affecting Y, β_0 is the constant coefficient, β_i is the linear coefficient, and β_{ij} is the coefficient interaction of two factors (X_i and, X_j) and, β_{ii} is a factor's quadratic coefficient (X_i^2). The BBD conducted 27 experiments, including three central points with two replicates for each experiment. Experiments were randomized, and all trial's response values were analyzed using Design-Expert (Version 11.1.0, Stat-Ease Inc., Minneapolis, MN, USA).

Table 1. BBD design for 4 factors and 3 levels for each factor.

Independent variable	Symbol	Factor level		
		(-1)	(0)	(+1)
Ultrasonic power (%)	X_1	60	80	100
The solid-liquid ratio (g/mL)	X_2	1:30	1:40	1:50
Extraction time (minute)	X_3	30	60	90
Extraction temperature (°C)	X_4	50	60	70

2.2.6. Statistical analysis

Each experiment was done thrice, and results were presented as means ± SD. Comparison of different samples was performed using t-test and ANOVA in the GraphPad Prism 9 software. The difference is considered significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Fatty acid composition in DO-R

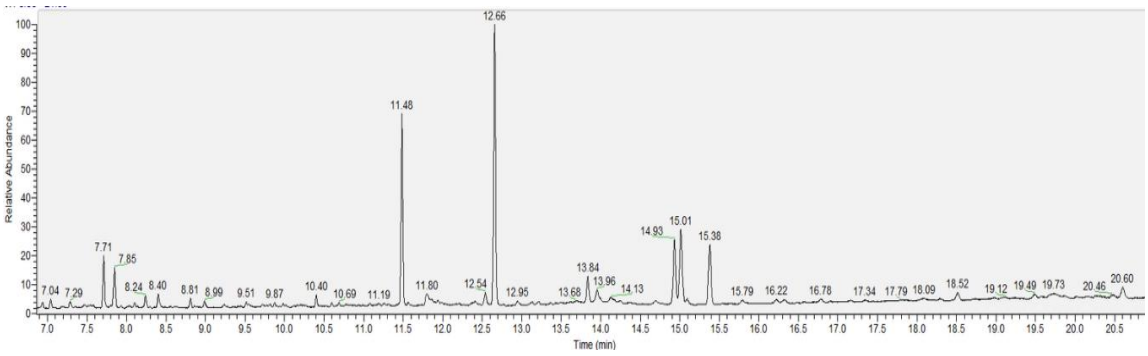

Figure 1. GC-MS chromatogram for fatty acids composition in DO-R.

Table 2. Composition of fatty acids in DO-R.

No.	Retention time (min)	Name of fatty acid	Formula	Composition (%)
1	7.29	Cis-10-heptadecenoic acid (C17:1)	$C_{17}H_{28}O_2$	1.62
2	9.51	10,13-octadecadiynoic acid (C18:4)	$C_{18}H_{28}O_2$	1.09
3	12.54	Arachidonic acid (C20:4)	$C_{20}H_{32}O_2$	3.97
4	12.65	Palmitic acid (C16:0)	$C_{16}H_{32}O_2$	44.53
5	13.96	Anteiso-heptadecanoic acid (C17:0)	$C_{17}H_{34}O_2$	2.37
6	14.93	Linoleic acid (C18:2)	$C_{18}H_{32}O_2$	14.80
7	15.01	Trans-13-octadecenoic acid (C18:1)	$C_{18}H_{34}O_2$	17.63
8	15.37	Linolelaidic acid (C18:2)	$C_{18}H_{32}O_2$	12.43
9	20.6	Eicosapentaenoic acid (EPA) or ω-3 fatty acid	$C_{20}H_{30}O_2$	1.56
Saturated fatty acids (**4**, **5**)				46.90
Unsaturated fatty acids (**1** – **3**, **6** – **9**)				53.10
Total				100 %

According to Fig. 1 and Table 2, nine fatty acids were contained in DO-R, in which unsaturated fatty acids (USFA) accounted for 53.1 %, and saturated fatty acids (SFA) were found with 46.9 %. Palmitic acid is the most abundant SFA, with 44.53 %, followed by anteiso-heptadecanoic acid with only 2.37 %. The three most important USFA in DO-R included trans-13-octadecenoic acid (17.63 %), linoleic acid (14.80 %) and linolelaidic acid (12.43 %).

Interestingly, the USFA: SFA ratio of DO-R in the current study is 1.13, which is a favorable ratio in foods and medicinal plants to reduce the risk of cardiovascular disease, according to Min *et al.* [23]. Especially primary fatty acids in DO-R have been documented to possess several pharmacological effects, including the antiviral effect of trans-13-octadecenoic acid [25] or the antioxidant and antibacterial activity of linoleic acid [25, 26]. Moreover, linoleic acid is an essential fatty acid favored in cosmetic products for its ability to soften and moisturize skin, hair, and nails [27]. Furthermore, the percentage of linoleic acid in DO-R was similar to that reported in Zingiber officinale rhizomes [28]. Noticeably, EPA, a long-chain omega-3 polyunsaturated fatty acid, was detected in DO-R with a relatively significant percentage (1.56 %). This kind of fatty acid was shown to bring many health benefits, such as preventing Alzheimer's disease [29], reducing the risk of cardiovascular diseases [30], inhibiting inflammatory processes [31], or treating several cancer cells [32].

3.2. Free amino acid content in DO-R

The profile of free amino acid in DO-R is shown in Figure 2 and Table 3.

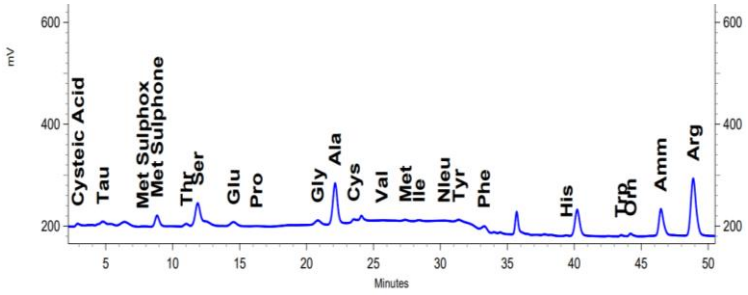

Figure 2. Chromatogram for free amino acids in DO-R.

Table 3. Free amino acid content in DO-R.

No	Amino acid	Symbol	Content (mg/100 g)	Percentage (%)
1	Threonine	Thr	2.60 ± 0.40	0.88
2	Valine	Val	23.68 ± 0.73	8.06
3	Methionine	Met	3.84 ± 1.38	1.31
4	Isoleusine	Ile	3.09 ± 0.60	1.05
5	Phenylalanine	Phe	5.87 ± 1.66	2.00
6	Histidine	His	0.80 ± 0.08	0.27
7	Tryptophan	Trp	1.93 ± 0.56	0.66
8	Taurine	Tau	15.09 ± 4.56	5.13
9	Serine	Ser	27.44 ± 4.23	9.34
10	Glutamic acid	Glu	9.39 ± 0.55	3.19
11	Proline	Pro	23.47 ± 2.25	7.99
12	Glycine	Gly	3.65 ± 0.74	1.24
13	Alanine	Ala	31.68 ± 3.25	10.78
14	Cysteine	Cys	10.74 ± 0.33	3.65
15	Tyrosine	Tyr	23.37 ± 6.57	7.95
16	Ornithine	Orn	3.33 ± 0.26	1.13
17	Arginine	Arg	103.94 ± 1.20	35.37
Total essential amino acids (**1** – **7**)			41.79 ± 1.02	14.22
Total non-essential amino acids (**8** – **17**)			252.09 ± 1.06	85.78
Total free amino acids			293.88 ± 2.08	100

The results showed that DO-R contained 17 free amino acids (FAA), of which seven essential amino acids (EAA) were detected with 14.22 %, and 10 non-essential amino acids (N-EAA) accounted for 85.78 % of the total FAA. In the EAA group, valine was the most abundant free amino acid which was found at 23.68 ± 0.73 mg/100 g (8.06 %), followed by phenylalanine (2.00 %) and methionine (1.31 %). These amino acids are also crucial in many foods and dietary supplements [33]. The N-EAA content ranged from 3.326 ± 0.264 to 103.94 ± 1.20 mg/100 g, in which the free amino acid with the highest content was arginine (35.37 %) and the lowest was ornithine (1.13 %). Arginine-rich proteins play a crucial role in many biological systems, such as gene expression, chromatin stability or pathogenesis defense, and exhibit several biological effects [34].

3.3. Total phenolic and flavonoid content

The TPC of different fractions from DO-R varied from 4.41 to 28.85 mg GAE/g dried DO-R, and the TFC range was between 2.37 and 14.04 mg QE/g dried DO-R (Table 4). For both parameters, the decreasing results were ME, EA, ET and DM. This finding suggests that methanol is the best solvent for extracting phenolic and flavonoid components in DO-R.

In a previous study, the TPC and TFC of the methanol extract of two *Z. officinale* varieties from Malaysia reached the maximum values of 13.5 mg GAE/g and 4.21 mg QE/g, respectively [35]. These results were remarkably minor than the values obtained for *D. orlowii* in the present study. However, the TPC and TFC of DO-R seem to be at lower values than the ones in the chloroform-methanol extract of *Z. officinale* in the study of Ali *et al.* [36].

Table 4. TPC and TFC values of DO-R extracts.

Extracts	TPC (mg GAE/g dried DO-R)	TFC (mg QE/g dried DO-R)
DM	4.41 ± 0.08^{a}	2.37 ± 0.07^{a}
EA	7.48 ± 0.53^{b}	7.94 ± 0.32^{b}
ME	28.85 ± 0.74^{c}	14.03 ± 0.31^{c}
ET	6.42 ± 0.16^{d}	5.88 ± 0.08^{d}

Different superscripts in each column refer to significant differences ($p < 0.05$).

3.4. Antioxidant activity

The antioxidative effect of DO-R extracts was assessed through their capacity to scavenge DPPH and ABTS radicals (Figure 3). For both radicals, the inhibitory effect was dose-dependent in a concentration range between 100 and 1000 µg/mL. In addition, the IC_{50} values varied considerably with different extracting solvents used. For the DPPH assay, ME was the most effective fraction with the lowest IC_{50} value (168.63 ± 4.83 µg/mL), followed by DM and ET. EA was shown to have the weakest antioxidant effect (IC_{50} = 342.23 ± 17.93 µg/mL). In contrast, ME (IC_{50} = 153.80 ± 8.24 µg/mL) and EA (IC_{50} = 144.53 ± 3.67 µg/mL) exhibited a more vital antioxidative activity against ABTS radical than other extracts ($p < 0.05$).

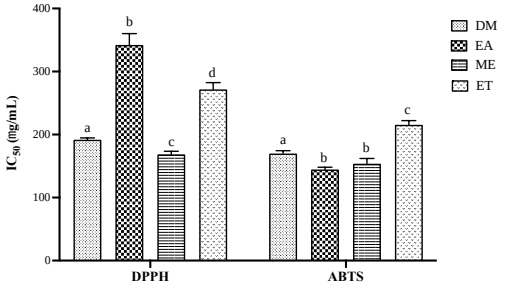

Figure 3. Antioxidant activity of DO-R extracts.

In comparison to the results obtained by Ali *et al.*, DO-R seems to be more effective than the extracts of *Z. officinale* rhizomes (IC_{50} = 342.23 ± 17.93 µg/mL) when scavenging ABTS radical [36]. Similarly, the antioxidative effect of DO-R extracts was considerably more potent than ginger *Zingiber officinale* fractions against DPPH radical (IC_{50}: 2.81 to 5.57 mg/mL) [37].

3.5. Optimization of the DO-R's extraction yield

3.5.1. The effect of a single factor on the extraction yield of DO-R

Ultrasonic power

Figure 4a shows that the extraction yield rose proportionally when the ultrasonic power ranged from 30 % to 80 %, and the yield achieved the maximum value (62.825 mg/g) at 80 % ultrasonic power. However, the extraction yield was reduced when the ultrasonic power was 100 %. Therefore, the range of variation of ultrasonic power used in the RSM experiments is 60 (level -1), 80 (level 0), and 100 % (level +1).

Solid-liquid ratio

As demonstrated in Figure 4b, the extraction yield progressed dramatically from 30.015 mg/g at 1:10 g/mL to a maximum of 61.850 mg/g at 1:40 g/mL. After that, the extraction mass decreased to 59.595 mg/g at 1:50 g/mL. Therefore, the solid-liquid ratio used in the subsequent experiments was set to 1:30, 1:40, and 1:50 g/mL for 3 levels of -1, 0, and +1, respectively.

Extraction time

The influence of extraction time on the extraction yield of DO-R is shown in Figure 4c. The mass of extract grew slightly when the extraction time increased from 20 to 60 min, before decreasing at 90 min. The maximum value (64.075 mg/g) was reached at 60 min extraction. Therefore, the extraction time of the experiments in RSM was from 30 to 90 min, respectively -1 and +1 levels, and 60 min was chosen as the central point (level 0).

Extraction temperature

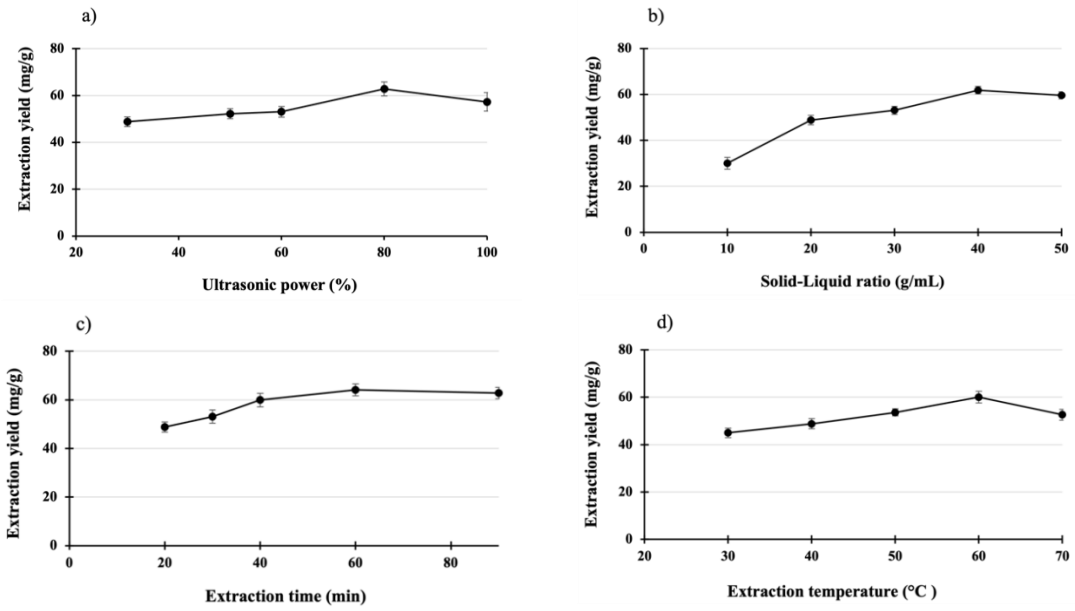

Figure 4. Effect of the single factor on the DO-R's extraction yield: a) Ultrasonic power, b) Solid-liquid ratio, c) Extraction time, and d) Extraction temperature.

The extraction yield increased from the lowest (44.970 mg/g) at 30 °C to the highest value (60.000 mg/g) at 60 °C, then decreased to 52.605 mg/g as the temperature rose to 70 °C (Figure

4d). As a result, in the RSM process, an extraction temperature of 50, 60, and 70 °C for 3 levels - 1, 0, and +1, respectively, were chosen.

Three levels for each factor of the experimental design were summarized in Table 1, then a matrix of experiments based on these levels was construct and analyzed in the next part of this study.

3.5.2. Optimization of variables by Box-Behnken design (BBD)

Statistical analysis and model fitting

Table 5. Design and result of BBD test.

Run	X_1	X_2	X_3	X_4	Y, extraction yield (mg/g)
1	100	40	90	60	83.035 ± 0.465
2	60	50	60	60	83.070 ± 0.550
3	80	30	60	70	80.375 ± 0.555
4	80	50	90	60	89.410 ± 0.830
5	60	30	60	60	84.705 ± 1.475
6	80	40	30	50	84.915 ± 0.115
7	60	40	60	50	69.105 ± 0.765
8	80	40	60	60	89.330 ± 0.490
9	80	30	90	60	85.000 ± 0.290
10	100	40	60	50	90.580 ± 0.090
11	80	40	60	60	91.375 ± 0.955
12	80	40	90	50	83.910 ± 2.530
13	100	30	60	60	77.855 ± 2.135
14	60	40	90	60	89.415 ± 1.215
15	60	40	30	60	83.465 ± 1.105
16	80	40	90	70	77.095 ± 0.525
17	80	50	30	60	93.770 ± 0.530
18	80	50	60	50	90.100 ± 0.380
19	80	40	60	60	90.335 ± 1.135
20	60	40	60	70	81.350 ± 0.150
21	80	40	30	70	78.760 ± 0.720
22	100	40	60	70	62.625 ± 0.105
23	80	30	30	60	85.610 ± 0.760
24	80	30	60	50	70.710 ± 0.070
25	100	50	60	60	88.205 ± 0.025
26	80	50	60	70	68.835 ± 0.185
27	100	40	30	60	92.150 ± 0.460

Table 5 shows how the experiments were randomized. Multiple regression analysis of the data from 27 experiments produced the quadratic polynomial equation 1 shown below:

$$Y = -789.32 + 4.49846X_1 + 6.04712X_2 + 0.511292X_3 + 18.96683X_4 + 0.014981X_1X_2 - 0.006277X_1X_3 - 0.05025\ X_1X_4 - 0.003125\ X_2X_3 - 0.077325\ X_2X_4 - 0.00055\ X_3X_4 - 0.010567X_1^2 - 0.027198X_2^2 + 0.000991X_3^2 - 0.101304X_4^2 \quad (1)$$

where Y, X_1, X_2, X_3, and X_4 represent the extraction yield, ultrasonic power, solid-liquid ratio, extraction time, and extraction temperature, respectively.

ANOVA was used to determine the model's significance and fit, and Table 6 summarizes the statistics. As can be seen, the model's F value is 124.497. The extremely low p-value ($p < 0.0001$) indicates that the model (1) is highly significant. The coefficient of determination (R^2) is the variation ratio in the data that the model explains or calculates. As a result, an R^2 of 0.993 is favorable. It demonstrates that this model can cover 99.32 % of the changes in response value, and its fitting accuracy is satisfactory. The adjusted coefficient (Adj. $R^2 = 0.985$) is close to R^2, indicating that the expected and experimental values agree. The variation coefficient (C.V. %) is small (1.17 %), meaning the model is accurate and reproducible. Linear variables X_2, X_3, and X_4 and quadratic variables X_1^2, X_2^2, and X_4^2 are statistically significant ($p < 0.01$); interactive two variables X_1X_2, X_1X_3, X_1X_4, and X_2X_4 have a remarkable influence ($p < 0.01$) on the extraction yield, whereas linear variables X_1, and interactive two variables X_2X_3, and X_3X_4, have no significant effects ($p > 0.05$) on the response.

Table 6. The quadratic polynomial mode's analysis of variance (ANOVA).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1636.310	14	116.800	124.497	<0.0001	Significant
X_1-Power	0.930	1	0.930	0.990	0.339	Not significant
X_2-Ratio	70.740	1	70.737	75.337	<0.0001	Significant
X_3-Time	9.730	1	9.729	10.362	0.007	Significant
X_4-Temperature	135.210	1	135.206	143.999	<0.0001	Significant
X_1X_2	35.910	1	35.910	38.245	<0.0001	Significant
X_1X_3	56.740	1	56.739	60.428	<0.0001	Significant
X_1X_4	404.010	1	404.010	430.282	<0.0001	Significant
X_2X_3	3.520	1	3.516	3.744	0.077	Not significant
X_2X_4	239.170	1	239.166	254.719	<0.0001	Significant
X_3X_4	0.109	1	0.109	0.116	0.739	Not significant
X_1^2	95.450	1	95.448	101.654	<0.0001	Significant
X_2^2	39.450	1	39.452	42.018	<0.0001	Significant
X_3^2	4.240	1	4.238	4.514	0.055	Not significant
X_4^2	547.340	1	547.335	582.927	<0.0001	Significant
Residual	11.270	12	0.939			
Lack of Fit	9.180	10	0.918	0.878	0.641	Not significant
Pure Error	2.090	2	1.046			
Cor Total	1647.580	26				
R^2	0.993					
Adjusted R^2	0.985					
Predicted R^2	0.965					
C.V %	1.17 %					

From the linear and quadratic coefficients, the selected factors influence the extraction yield in decreasing order as follows: extraction temperature > solid-liquid ratio > extraction time> ultrasonic power due to the decrease of F value (Table 6).

Optimization of the extraction yield

The regression function is represented graphically by three-dimensional contour plots (Figure 5). They showed the interaction of pairs between two variables. The 3D surface graphs in Figures 5a, 5b, 5c, and 5e have a curved shape showing the strong interaction between the two variables that the graphs represent, respectively. In contrast, Figures 5d and 5f show a 3D surface plot with a relatively flat shape showing an insignificant interaction between the solid-liquid ratio and extraction time (Figure 5d) and between extraction time and extraction temperature (Figure 5f). This was similar to our results from the ANOVA analysis (Table 6).

The optimal extraction parameters were obtained using the Design Expert version 11.0 software: an ultrasonic power of 80 %, a solid-liquid ratio of 1:40 g/mL, an extraction time of 60 min, and an extraction temperature of 58.342 °C. Under those factors, the approximated extraction yield was 95.625 mg/g. In the verification experiment, the optimal parameters were slightly modified for operational convenience: ultrasonic power of 80 %, a solid-liquid ratio of 1:40 g/mL, for 60 minutes at 58 °C. Recovery experimental results under optimal conditions confirmed the method's reliability. The results indicated that the observed value (95.534 ± 0.593 mg/g) was comparable with the predicted value (C.V % = 0.0673 %). This finding showed that the RSM experimental parameters are reliable, and the anticipated model can precisely forecast the optimum parameters of DO-R extraction.

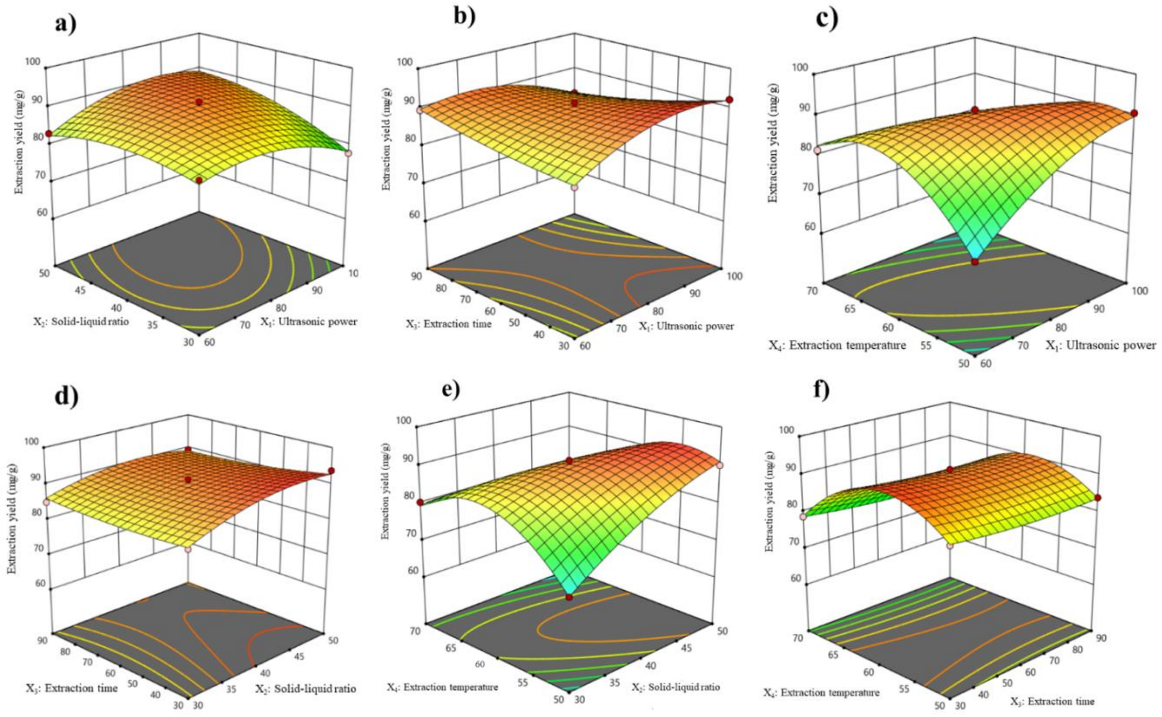

Figure 5. 3D model demonstrating the effect of the interaction between two various extraction parameters. X_1: ultrasonic power (%), X_2: solid-liquid ratio (mg/mL), X_3: extraction time (minute), and X_4: extraction temperature (°C).

4. CONCLUSIONS

DO-R was investigated for its chemical composition and antioxidant activity for the first time. GC-MS revealed nine fatty acids in DO-R, in which unsaturated fatty acids (USFA) dominated at 53.1% and saturated fatty acids (SFA) accounted for 46.9 %. Palmitic acid and trans-13-octadecenoic acid were the most abundant SFA and USFA, respectively. Among 17 free amino acids (FAA) found in DO-R, seven essential amino acids (EAA) were detected with 14.22 %, and 10 non-essential amino acids (N-EAA) took 85.78 % of the total FAA. The methanol extract from DO-R displayed the most potent antioxidative effect and had higher total phenolic and flavonoid contents than other extracts. The extraction yield of DO-R in methanol was optimized using response surface methodology combined with a Box-Behnken design. The highest response was obtained at 95.534 ± 0.593 mg/g DW when the extraction was performed under the optimal parameters, including the solid-liquid ratio (1: 40 g/mL), ultrasonic power level (80 %), extraction time (60 minutes), and extraction temperature (58 °C). In conclusion, DO-R is a potential source of phytoconstituents of great biological interest. Therefore, further investigations should be carried out to explore the health benefits of this plant in the future.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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