Hindawi
Journal of Food Biochemistry
Volume 2023, Article ID 6065283, 12 pages
https://doi.org/10.1155/2023/6065283

Research Article

Evaluation of the Potential of Duckweed as a Human Food, Bioethanol Production Feedstock, and Antileukaemia Drug

Ling Guo,[1] **Jing Liu**,[1] **Qingyan Wang**,[2] **Yan Yang**,[1] **You Yang**,[1] **Qulian Guo**,[1] **Hai Zhao**,[3] **and Wenjun Liu**[1]

[1]*Department of Pediatrics, Children Hematological Oncology and Birth Defects Laboratory, The Affiliated Hospital of Southwest Medical University, Sichuan Clinical Research Center for Birth Defects, Luzhou, Sichuan 646000, China*
[2]*State Key Laboratory of Non-Food Biomass and Enzyme Technology, Nanning, Guangxi 530000, China*
[3]*CAS Key Laboratory of Environmental and Applied Microbiology, Environmental Microbiology Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan 610041, China*

Correspondence should be addressed to Ling Guo; guoling@swmu.edu.cn, Hai Zhao; zhaohai@cib.ac.cn, and Wenjun Liu; wenjun_liu@swmu.edu.cn

Received 6 December 2022; Revised 6 February 2023; Accepted 17 March 2023; Published 27 March 2023

Academic Editor: Rotimi Aluko

This study evaluated the potential of duckweed as a human food, ethanol feedstock, and anticancer drug. First, the nutritional value of wild duckweed was reported for the first time. Its main composition was similar to that of artificially cultivated duckweed, and thus, wild duckweed can serve as a great human food source. In addition, high-starch duckweed induced by nutrient starvation was fermented into bioethanol. A yield of 0.262 g/g, the highest duckweed-ethanol yield reported thus far, was achieved, indicating that duckweed is an excellent feedstock for ethanol production. Finally, the anticancer effects of duckweed flavonoids (DFs) were assessed for the first time using acute myeloid leukaemia (AML) cells as models *in vitro* and *in vivo*. The results revealed that DFs possessed antileukaemia activity and were safe and effective for AML therapy. In conclusion, duckweed was demonstrated to be helpful for humans for food security, energy crisis remediation, and tumour treatment.

1. Introduction

Food security, energy crises, and tumours are the three major challenges that threaten human society worldwide. Studies on novel food alternatives, renewable energy, and anticancer drugs have thus become hotspots in recent years. However, the current research mostly focuses on only one particular field, although investigating a combination of multiple fields may be more promising to solve the abovementioned three problems.

Recently, duckweed, a globally distributed aquatic plant, has received increasing interest due to its intrinsic advantages, such as its rapid growth rate and lack of competition for arable land [1]. First, duckweed has been considered a human food source owing to its high content of protein, beneficial pigments, and sufficient source of essential amino acids recommended by the World Health Organization [2–6]. However, the duckweeds reported in previous studies were artificially cultivated in laboratory medium or semi-artificially cultivated in sewage; the nutritional value of wild duckweed has rarely been reported. In our opinion, the scale of artificially cultivated duckweed is far lower than that of wild duckweed worldwide, and wild duckweed is the major source for duckweed biomass production. Thus, the nutritional value of wild duckweed deserves to be systematically studied. In addition, duckweed has been identified as a potential bioenergy crop [7] due to its high starch accumulation capability [8]. Duckweed biomass has been reported as an industrial feedstock for the production of glycerol, 1,3-propanediol [9], succinic acid [10], and

especially, bioethanol, an acknowledged alternative to fossil energy [11]. However, the duckweed-ethanol conversion rate is still low, and the fermentation process needs to be further optimized. Finally, duckweed has been used as a folk medicine for a long time in China [12]. It possesses various pharmaceutical activities, such as antibacterial, antifungal, antioxidant, immunomodulatory, and antiadipogenic effects [13]. However, the anticancer effect of duckweed has not been elucidated, and its further investigation is therefore essential.

In this study, the main composition of wild duckweed (mainly *Landoltia punctata*) was determined, and its nutritional value was systematically evaluated for the first time. In addition, to improve the bioethanol yield, high-starch duckweed biomass induced by nutrient starvation was fermented into ethanol using separate hydrolysis and fermentation. The annual duckweed-to-ethanol yield was estimated according to the annual yield of duckweed biomass. Finally, the anticancer effects of duckweed were identified *in vitro* and *in vivo* using acute myeloid leukaemia cells as models, as this disease is a common refractory haematological malignancy in children with ease of relapse.

2. Materials and Methods

2.1. Plant Material and Cultivation. Wild duckweed (*Landoltia* ≥90%, *Spirodela* 3–5%, *Lemna* 2–4%, *Wolffia* ≤1%, and without *Wolffiella*) was directly harvested from ponds and paddies across the cities in Sichuan Province. The harvested duckweed materials were rinsed with tap water until the impurities were negligible. A part of the duckweed was used for component determination, and the other parts were cultivated under nutrient deficiency to accumulate starch and flavonoids for subsequent experiments. The cultivation conditions were as follows: the fronds were seeded in tap water in 500 mL plastic beakers (9.0 cm outer diameter × 12.0 cm tall); photoperiod of 16 h/8 h (day/night); light intensity of 130 μM photons/m^2/s; temperature of 25°C/20°C (day/night); cultivation time of 10 d; and the sampling time points were at 0, 3, and 10 d. The growth rate (GR) based on dry matter was calculated as follows:

$$\mathrm{GR}\left(\frac{g/m^2}{d}\right) = \frac{W_1 - W_0}{s \times t} \times 100, \quad (1)$$

where W_0 (g) is the initial dry weight of the sample, W_1 (g) is the final dry weight of the sample (g/m^2), s is the cultivation area (m^2), and t is cultivation time (d).

2.2. Duckweed Composition. Fresh duckweed and dry duckweed powder were applied to determine the main components of duckweed. First, fresh duckweed samples were dried at 105°C to a constant weight, and moisture was calculated as follows:

$$\text{Moisture content } (\%) = \frac{W_{\text{fresh}} - W_{\text{dry}}}{W_{\text{fresh}}} \times 100. \quad (2)$$

The carbon (C%) and nitrogen contents (N%) were determined by an elemental analyser (Elementar Analysensysteme, Vario Macro Cube, C/N mode, TCD, Germany), and the crude protein content was calculated as 6.25 × N%. The duckweed starch content was determined according to the method of Yin et al. [14] with some modification. In brief, 30 mg of dry powder was accurately weighed and transferred to a 10°mL tube. Then, 2.0 mL of distilled water was added. After sonication at 70°C for 30 min, the mixture was centrifuged at 12,000 rpm for 10 min, and the supernatant was discarded. This step was repeated twice. Then, 1.0 mL of distilled water and 1 μL of thermostable α-amylase (Novozymes, China) were added. The mixture was sonicated at 90°C for 45 min. After cooling, 1.0 mL of Na acetate buffer (200 mM, pH 4.8) and 1 μL of glucoamylase (Suhong GAII, Novozymes, China) were added. The mixture was maintained at 60°C for 2 h of hydrolysis and centrifuged for 10 min at 12,000 rpm. Thereafter, the supernatant was filtered and analysed by HPLC (Thermo 2795, Thermo Corp.) with an evaporative light scattering detector (All-Tech ELSD2000, All-tech Corp.). The starch content was determined using the total sugar content (starch content = glucose content × 0.909). The soluble sugar content was measured according to Gao et al. [15]. A dry sample of duckweed (30 mg) was accurately weighed and placed in a 10°mL EP tube. Two millilitres of distilled water was added and the solution was sonicated for 30 min. Then, the mixture was incubated in a water bath at 85°C, and the supernatant was collected by centrifugation. This step was repeated twice, and then distilled water was added to a final volume of 10 mL. The soluble sugar content was determined by sulfuric acid-anthrone colorimetry at 620 nm. Ash and amino acid composition were assayed according to the methods described by Hu et al. [4].

2.3. Duckweed Flavonoid Extraction and Analysis. Duckweed flavonoids (DFs) were extracted according to the methods described by Tsolmon et al. [16] with some modification. In brief, 100 g of duckweed powder was extracted with 2.0 L of 70% (v/v) methanol by three rounds of sonication (30 min each). The mixture was centrifuged at 12,000 rpm. The precipitate was collected and dried in an oven at 60°C for subsequent bioethanol fermentation. The supernatant was combined and concentrated with a rotary evaporator under reduced pressure at 60°C. The concentrate was then lyophilized to a dry powder, 0.1 g of which was accurately weighed and dissolved in 10 mL of 70% methanol. Finally, the solvent was filtered through a PTFE membrane syringe filter (0.22 μm) prior to use. The flavonoid content was analysed by HPLC (AS3000 Auto Sampler, Thermo Electron Corp, USA)-UV (UV6000 DAD Detector, USA) using luteolin-7-O-glucoside as a standard [17]. The mobile phase consisted of methanol (A) and deionized water supplemented with 0.5% (v/v) acetic acid (B). Additional HPLC parameters are as follows: detection wavelength: 326 nm; flow rate: 0.6 mL/min; column: Kromasil 100-5C18 (250 mm × 4.6 mm, 5 μm); column temperature: 35°C; gradient elution procedure: 0–20 min

30–40% A, 20–30 min 40–53% A, 30–40 min 53–90% A, and 40–45 min 90% A.

2.4. Starch Hydrolysis. Duckweed starch was hydrolysed using the two-step enzymatic hydrolysis method described by Perez-Rea et al. [18]. In brief, 19 g of duckweed powder (starch content = 50.8%) was mixed with 110 mL of distilled water in a 250°mL glass Erlenmeyer flask. The mixture was sonicated for 30 min, and 10 μL of liquefaction enzymes (Liquozyme Supra, Novozymes, China) were added. The mixture was incubated at 100°C for 1 h. After adjusting the pH to 4.5, 10 μL of saccharification enzymes (Suhong GAII, Novozymes, China) were added. Then, the mixture was maintained at 60°C for 2 h. Finally, the duckweed mash was autoclaved at 115°C for 20 min. The sterile mash was weighed and stored at room temperature prior to use.

2.5. Ethanol Fermentation. Ethanol fermentation was carried out according to Economou et al. [19]. *Saccharomyces cerevisiae* (CCTCC M206111) strains (isolated from wine lees in our laboratory) were added to the sterile mash at a 10% v/w ratio and fermented at 30°C for 24 h. Then, 2.5 g of fermentation mash was weighed and diluted with distilled water to a final volume of 25 mL. The suspension was centrifuged at 13,400 × g for 10 min, and the supernatant was filtered for ethanol content determination. The ethanol content was determined by gas chromatography (GC, Agilent Technologies 7820A, California, USA) [20].

The ethanol conversion rate ($Y_{E/G}$), ethanol fermentation efficiency (%), and ethanol yield based on the duckweed biomass input ($Y_{E/B}$) were calculated as follows [21]:

$$Y_{E/G}\ (g/g) = \frac{Y_E}{M_B \times S_D \times 1.1}.$$

$$\text{Ethanol fermentation efficiency}\ (\%) = \frac{Y_{E/G}}{0.511} \times 100,$$

$$Y_{E/B}\ (g/g) = \frac{Y_E}{M_B}, \tag{3}$$

where Y_E (g) is the total ethanol yield in the final fermentation mash, M_B (g) is the initial duckweed biomass input, S_D (%) is the duckweed starch content, and 0.511 is the theoretical ethanol conversion rate.

2.6. Cell Lines and Cell Culture. Two human AML cell lines, HL60 and Thp1 (Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology), were cultured at 37°C in 5% CO_2 in RPMI 1,640 medium (Gibco, Beijing, China) containing fetal bovine serum (10%, Biological Industries, Israel), penicillin (100 IU/mL), and streptomycin (100 μg/mL, Gibco, NY, USA).

2.7. Cell Viability Assay. CCK8 kits were used to evaluate cell viability [22]. In brief, 2.0×10^4 cells were seeded in a 96-well culture plate. Then, different concentrations of DFs were added. After 24 h of treatment, cell viability was determined by CCK8 assay according to the manufacturer's instructions (Beyotime, Shanghai, China).

2.8. Apoptosis Assay. HL60 and Thp1 cells exposed to different concentrations of DFs were collected and incubated in the dark at 37°C in 1 mL of fresh medium containing 1 μg/mL Hoechst 33,258 for 30 min. Subsequently, the cells were washed twice with PBS and monitored by fluorescence microscopy (OLYMPUS IX73 Microscope, Tokyo, Japan).

Apoptotic rates were determined using an apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA) [23]. First, 2.5 mL of cells (5.0×10^5 cells) were seeded in 6-well culture plates and exposed to DFs (20, 40, and 60 μg/mL for HL60 cells; 40, 60, and 80 μg/mL for Thp1 cells). After 24 h of treatment, the cells were collected and resuspended in 500 μL of 1 × Annexin V binding buffer. Then, 5 μL of Annexin V-FITC and propidium iodide were added in turn. Cells were incubated in the dark at RT for 15 min. Finally, apoptotic cells were quantified by flow cytometry (BD FACSVerse flow cytometer, San Diego, CA).

2.9. Western Blot Analysis. Treated cells were lysed in precooled RIPA buffer containing protease and phosphate inhibitors (Roche, Mannheim, Germany). The protein concentration was quantified by a Bradford dye-binding assay. Then, 30 μg of protein was separated by SDS-PAGE and transferred to a PVDF membrane. Subsequently, the membranes were incubated with the primary antibodies at 4°C overnight. After three washes with PBST, the membranes were incubated with the secondary antibodies at RT for 1 h. Finally, the proteins were visualized by an enhanced chemiluminescence system (Beijing 4A Biotech Co. Ltd., China) [24].

2.10. In Vivo Animal Experiments. All animal experiments were approved by the Ethics Committee of Southwest Medical University (protocol number: 20211019-002). Healthy BALB/c-nu/nu mice (female, 4-5 weeks old) were purchased from Huafukang Biotech. Co. Ltd. HL60 or Thp1 cells (5×10^6) were injected subcutaneously into the right lower limb of each mouse. When the average tumour volume reached approximately 100 mm^3, the mice were randomly divided into treatment and control groups with five mice in each group, and the mice were intragastrically injected with 60 mg/kg DFs and vehicle, respectively. Tumour volume was measured every 2 days. The tumour volume was calculated as follows: tumour volume $(mm^3) = (\text{length}) \times (\text{width})^2 \times 0.5$. At the end of treatment, the tumours were dissected, imaged, and weighed.

2.11. Statistical Analysis. Three independent repeats were performed in all experiments in this study. SPPS software (version 21.0) and GraphPad Prism (version 8.0) were applied for statistical tests. One-way ANOVA and unpaired t tests were used to analyse the statistical significance of

differences among the different groups. $p < 0.05$ was considered significant.

3. Results

3.1. Main Composition. As presented in Table 1, the dry weight of wild duckweed was approximately 8% of the fresh weight. Based on the dry weight, the contents of the other components were determined. First, the carbon content was 38.77%, and the nitrogen and crude protein contents were 3.97% and 24.80%, respectively. Second, the starch and soluble sugar contents reached 8.87% and 1.44%, respectively. Moreover, the ash content was 8.97%, indicating abundant mineral contents. Finally, to further evaluate the nutritional quality of wild duckweed, the amino acid composition of the total protein was determined. As shown in Table 1, 17 amino acids were found in duckweed, and the total amino acid (TAA) content was 7.5% of the dry weight. Among these amino acids, essential amino acids (EAAs) accounted for 41% of the TAAs, while nonessential amino acids (NEAAs) accounted for 59%, which were more abundant than the EAAs. The abovementioned results indicated that duckweed possessed potential as a food feedstock.

3.2. Starch and Flavonoid Accumulation. To promote starch and flavonoid accumulation, duckweed was cultivated in tap water (nutrient starvation) for 10 days. The results are shown in Figure 1. First and foremost, the biomass increased continuously from the original 15.04 ± 0.62 to 72.53 ± 2.78 g/m^2 on the 10th day, achieving a mean growth rate of 5.75 ± 0.29 g/m^2/d. In addition, starch accumulation was tested during the cultivation process. As shown in Figures 1(c) and 1(d), the starch content significantly increased from 8.97% to 36.35% DW in 3 days. The highest starch content reached 51.45% DW on the 10th day, which was 5.74 times higher than that on the first day. Finally, the flavonoids were extracted and quantified. As illustrated in Figures 1(e) and 1(f), the flavonoid content exhibited an increasing trend. The initial flavonoid content was 1.81 ± 0.22% DW, which then gradually increased to 2.33 ± 0.03% DW on the 3rd day. Thereafter, the flavonoid content changed insignificantly to 2.53 ± 0.12% DW on the 10th day. The flavonoid extract was concentrated and stored at −20°C. The residues after flavonoid removal were then used for bioethanol fermentation.

3.3. Bioethanol Fermentation. To determine whether high-starch duckweed can serve as a feedstock for industrial production, the quality of duckweed biomass should be evaluated. In this study, we converted duckweed biomass into ethanol using separate hydrolysis and fermentation (SHF). After 24 h of SHF, the mean ethanol yield reached 4.98 ± 0.06 g in each Erlenmeyer flask, achieving an ethanol productivity of 0.208 ± 0.002 g/h. As shown in Table 2, the ethanol conversion rate ($Y_{E/G}$) reached 0.469 g/g glucose, and 91.83% fermentation efficiency was achieved. In addition, the ethanol yield ($Y_{E/B}$) under SHF reached 0.262 g/g biomass; based on the biomass yield (26.11 t/ha/y), a potential volume of duckweed-bioethanol of 8670 L/ha/y can be produced (Table 3).

3.4. The Antileukaemic Effect of Duckweed Flavonoids In Vitro

3.4.1. Cell Viability. To confirm the antileukaemic effect of duckweed flavonoids (DFs), the cytotoxicity of the DFs was first measured. Two AML cell lines (HL60 and Thp1) were exposed to different concentrations of DFs (10, 20, 40, 60, 80, and 100 μg/mL). After 24 h of treatment, the viability of both cell lines showed a sharp decrease with increasing DF concentration, indicating that DFs inhibited the growth of AML cells in a dose-dependent manner (Figure 2(a)). Furthermore, the DFs gave IC_{50} values of 31.35 ± 4.45 μg/mL against HL60 cells and 62.93 ± 7.73 μg/mL against Thp1 cells (Figure 2(b)). Based on these IC_{50} values, 0, 20, 40, and 60 μg/mL DFs were chosen to treat HL60 cells, while 0, 40, 60, and 80 μg/mL DFs were chosen to treat Thp1 cells in the subsequent experiments.

3.4.2. Apoptosis. After the cell viability assay, apoptosis was detected. First, apoptosis was morphologically monitored using an inverted fluorescence microscope. The results displayed clear apoptotic cells of both lineages after DF treatment (Figures 2(c) and 2(d)). The number of apoptotic cells increased as the DF concentration increased, which indicated that DFs can induce apoptosis in AML cells in a dose-dependent manner. Then, apoptosis was quantified by flow cytometry. The changes in the apoptotic rates in both cell lines were in good agreement with the morphological results (Figures 2(c) and 2(d)). The apoptotic rate significantly increased in both types of treated cells. The highest apoptotic rates of 76.31 ± 3.79% and 38.08 ± 16.95% were observed in HL60 cells in the 60 μg/mL treatment group and in Thp1 cells in the 80 μg/mL treatment group, respectively. Finally, DF-induced apoptosis was further demonstrated at the protein expression level. The Western blot results showed clear increases in cleaved PARP and cleaved caspase 3 expression levels after DF treatment (Figure 2(e)), which confirmed that DFs can indeed induce apoptosis in HL60 and Thp1 cells *in vitro*.

3.5. The Antileukaemic Effect of Duckweed Flavonoids In Vivo. To further evaluate the antileukaemic effect of the DFs *in vivo*, AML cell-xenografted nude mice (5×10^6 HL60 or Thp1 cells/mouse) were intragastrically administered 60 mg/kg DFs per day for 12 days. As shown in Figures 3(a) and 3(b), DF administration significantly inhibited the growth of the tumours from both AML cell lines. At the end of treatment, the wet tumour weights in the DF treatment groups were 1.57 ± 1.06 g (HL60-xenograft tumours) and 2.65 ± 0.88 (Thp1-xenograft tumours). Compared to the control groups (4.27 ± 1.35 g, HL60-xenograft tumours and

Table 1: Main components of wild duckweed.

Duckweed	Content	Essential amino acids (% DW)		Nonessential amino acids (% DW)	
Moisture (% FW)	91.74 ± 0.47	Threonine	0.38 ± 0.02	Aspartic acid	0.91 ± 0.17
C% (% DW)	38.77 ± 0.26	Valine	0.45 ± 0.01	Serine	0.38 ± 0.02
N% (% DW)	3.97 ± 0.32	Methionine	0.15 ± 0.00	Glutamic acid	0.93 ± 0.07
C/N	9.77 ± 0.08	Isoleucine	0.34 ± 0.01	Glycine	0.45 ± 0.02
Crude protein (% DW)	24.80 ± 0.36	Leucine	0.71 ± 0.03	Alanine	0.53 ± 0.03
Starch (% DW)	8.97 ± 1.88	Phenylalanine	0.45 ± 0.01	Cystine	0.10 ± 0.01
Soluble sugar (% DW)	1.44 ± 0.09	Lysine	0.47 ± 0.02	Tyrosine	0.32 ± 0.02
Ash (% DW)	8.97 ± 0.01	Histidine	0.15 ± 0.01	Arginine	0.48 ± 0.02
TAAs (% DW)	7.50 ± 0.44			Proline	0.34 ± 0.01

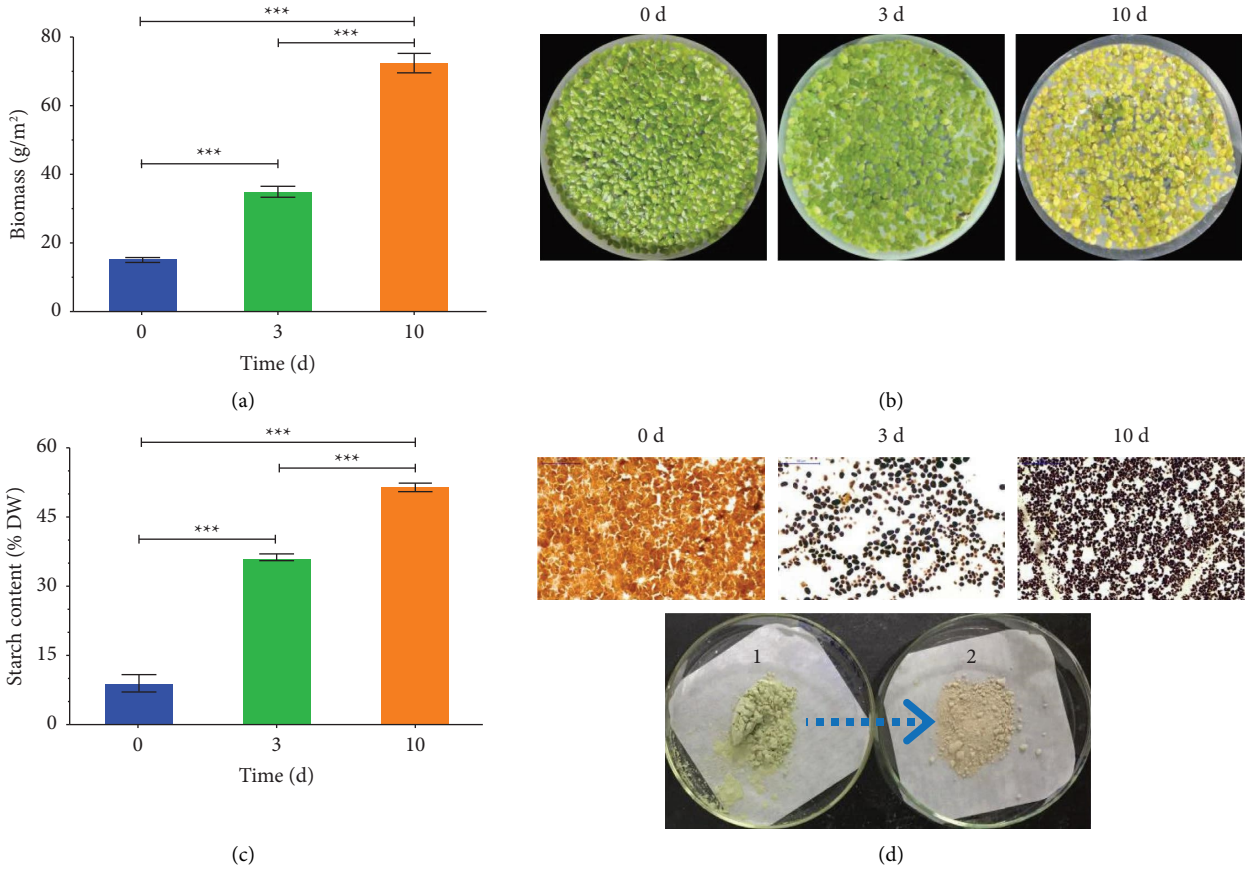
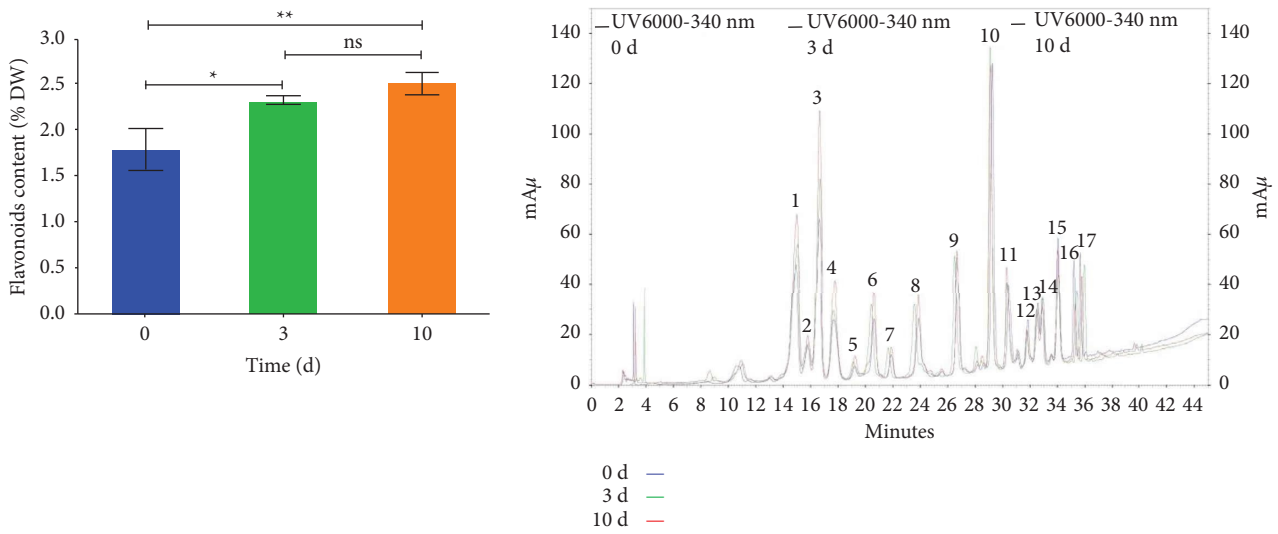


Figure 1: Continued.



(e) (f)

FIGURE 1: Changes in biomass and starch and flavonoid contents in duckweed: (a, b) duckweed biomass, (c) starch content, (d) morphology of the starch granules in the duckweed fronds: (1) high-starch duckweed biomass before flavonoid removal and (2) high-starch duckweed biomass after flavonoid removal, and (e, f) changes in flavonoid contents in duckweed. ns: not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control.

TABLE 2: Separate hydrolysis and fermentation (SHF) of high-starch duckweed for ethanol production.

Biomass hydrolysis		Ethanol fermentation	
Biomass input (g)	19.0	Ethanol content (g/g fermentation mash)	0.0363 ± 0.0000
Starch content (%)	50.8 ± 1.4	Total ethanol yield (g)	4.98 ± 0.05
Total glucose input (g)	10.62	Ethanol productivity (g/h)	0.208 ± 0.002
Weight of final fermentation mash (g)	137.21 ± 0.26	Ethanol fermentation efficiency (%)	91.82 ± 0.96
		$Y_{E/G}$ (g/g glucose)	0.469 ± 0.005
		$Y_{E/B}$ (g/g biomass)	0.262 ± 0.003

TABLE 3: Comparisons of the yield, starch yield, and potential bioethanol production between the major bioethanol cereal crops and duckweed.

Parameters	Crops		
	Wheat (grain)	Maize (kernel)	Duckweed (whole plant)
Average world yield (t/ha/y)	3.47[a]	5.75[a]	26.11[#]
DW of starch yield (t/ha/y)	2.42[b]	4.03[c]	13.43[#]
Potential yield of bioethanol (L/ha/y)	1552[d]	2585[d]	8670[#]

[a]FAOSTAT [25], https://www.fao.org/faostat/en/#data/QC; [b]Zhou et al. [26]; [c]Wang et al. [27]; [d]assuming an ethanol conversion rate of 0.46 according to Adams et al. [28].

5.13 ± 0.72 g, Thp1-xenograft tumours), the inhibition rates reached 63.33% and 48.37%, respectively. These results indicated that 60 mg/kg DFs can efficiently suppress the growth and reduce both the volume and weight (Figures 3(a)–3(c)) of tumours of these two AML cell lines. Regarding the inhibitory effects, HL60 cells were more sensitive to DFs than Thp1 cells, which were consistent with the results of the *in vitro* experiment. Finally, haematoxylin and eosin (H&E) staining of tumour tissues reconfirmed that DFs can efficiently induce AML cell death (Figure 3(d)). In summary, these results revealed that DFs possessed powerful antileukaemia activity *in vitro* and *in vivo*.

4. Discussion

4.1. The Potential of Duckweed as a Novel Food Feedstock. Reportedly, nearly 690 million people were suffering from hunger in 2019, accounting for 8.9% of the world's population [25]. It is estimated that the world's population will reach 9 billion by mid-century, which will cause more severe

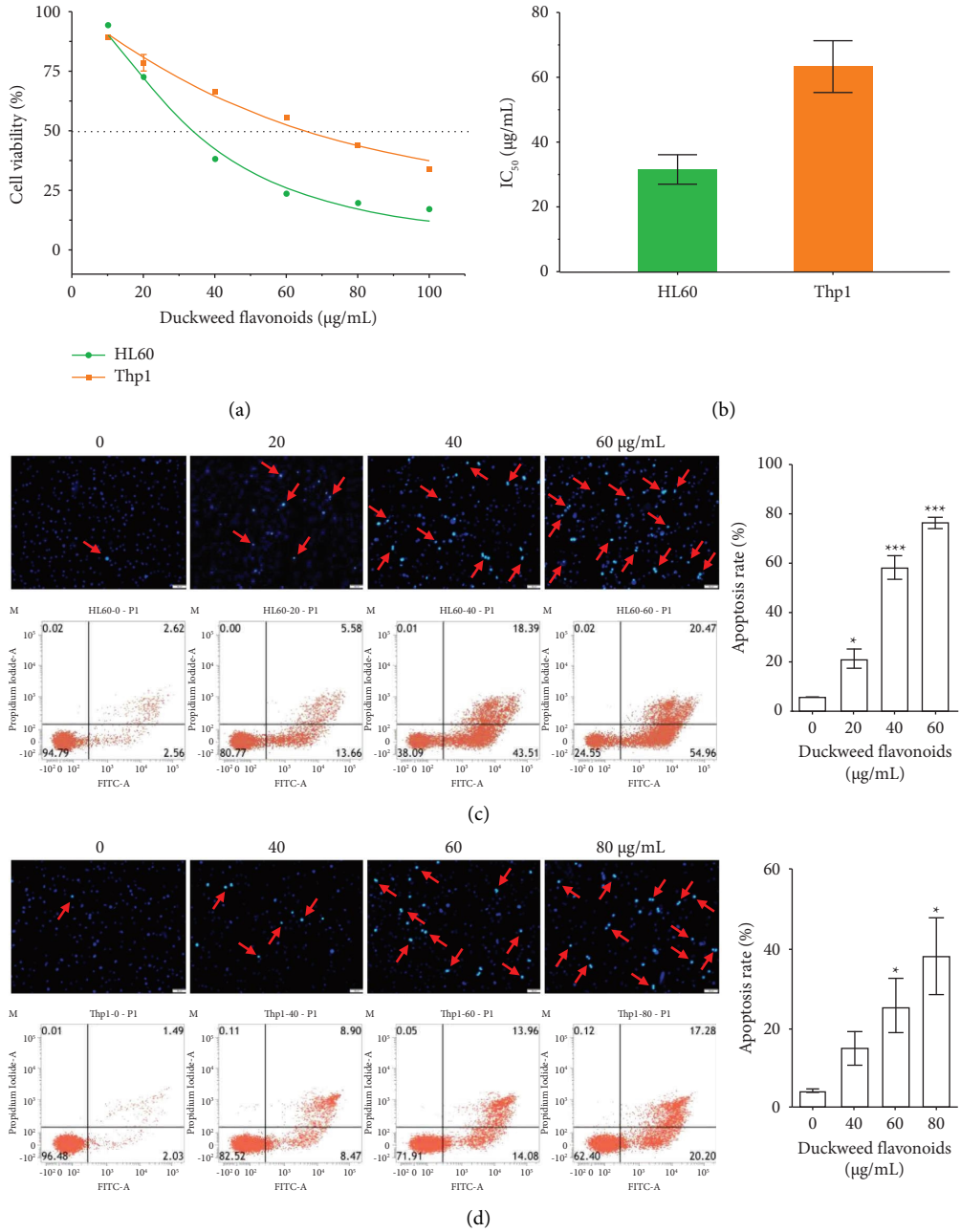
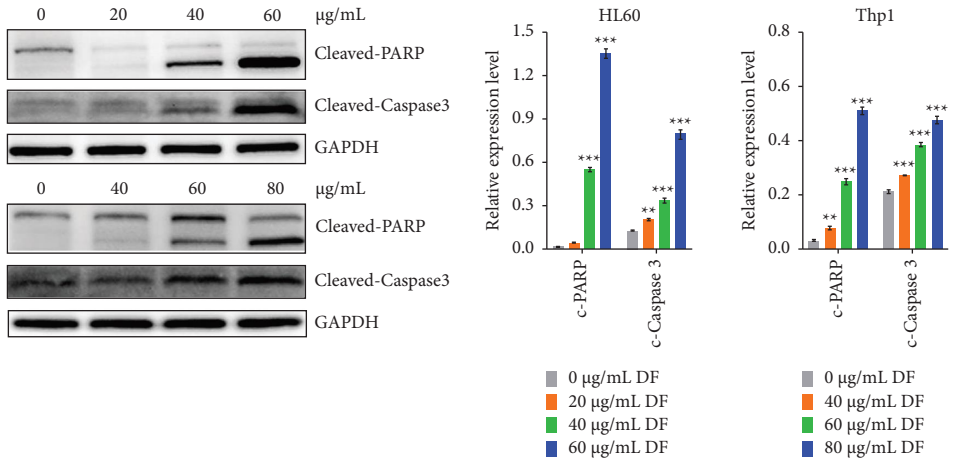
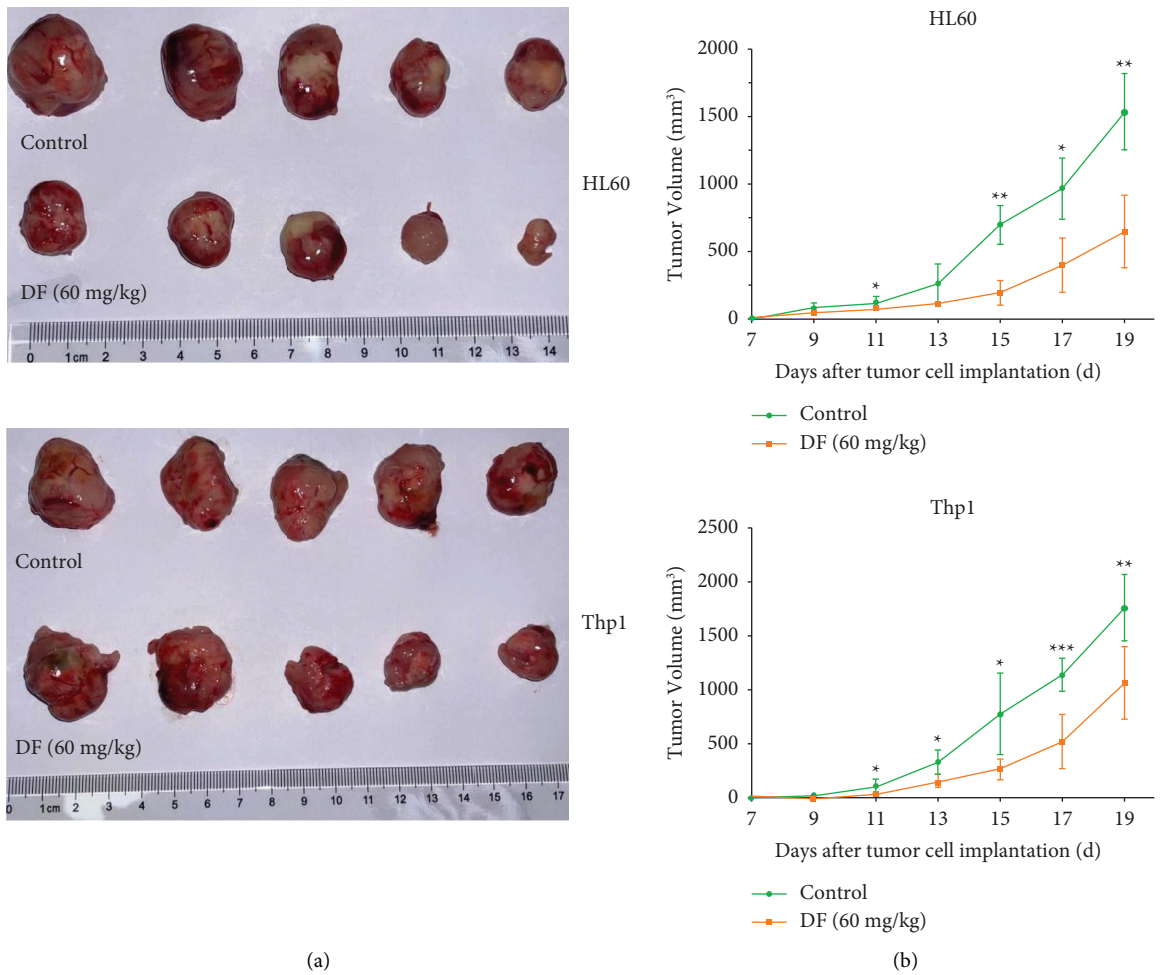


Figure 2: Continued.



(e)

FIGURE 2: The antileukaemic effects of duckweed flavonoids *in vitro*: (a) cell viability after DF treatment, (b) DF IC_{50} values, (c, d) apoptosis of HL60 and Thp1 cells, and (e) the levels of apoptosis-related proteins determined by Western blot analysis. GAPDH was used as the loading control. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control.



(a) (b)

FIGURE 3: Continued.

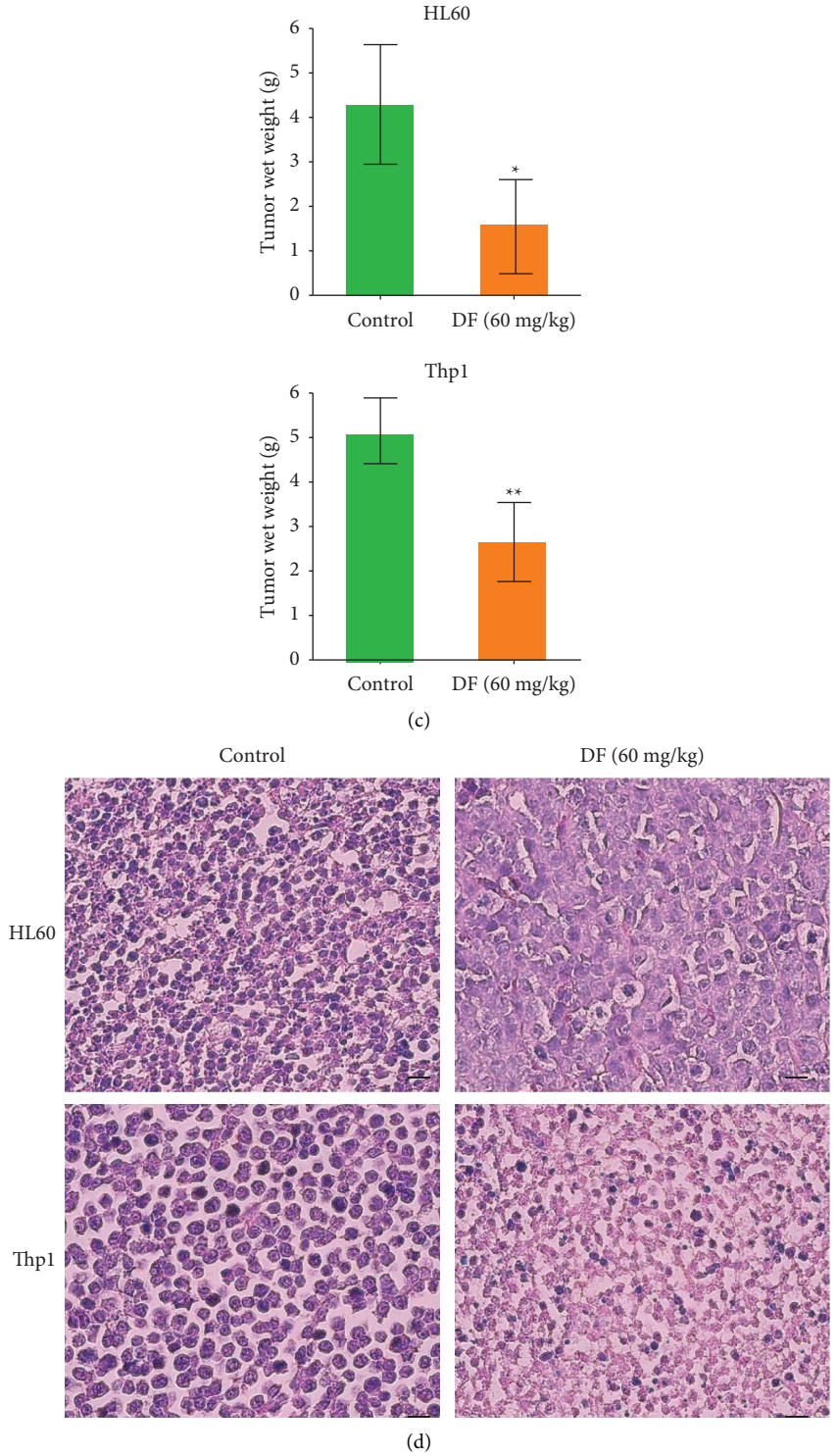


Figure 3: The antileukaemic effects of duckweed flavonoids *in vivo*: (a) tumour images, (b) changes in tumour volume, (c) tumour weights, and (d) tumour sections stained with H&E. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control.

food insecurity. Due to the limited amount of arable land and bottlenecks in traditional crop cultivation technology worldwide, novel aquaculture crops have been proposed as important additions to the global food system [29]. Hu et al. [4] and Appenroth et al. [30] determined the nutritional value of *Wolffia*. Similarly, Pagliuso et al. [5] found that duckweed can serve as a promising food source worldwide. Furthermore, Appenroth et al. [2] reported that the nutritional component distribution of duckweed was close to the WHO recommendations. However, all of the duckweeds in

these studies were cultivated under artificial conditions, and the nutritional value of natural duckweed is still undetermined. In this study, we first reported the main composition of natural duckweed. As the biggest concern for food nutrition, the protein and TAA contents of natural duckweed were 24.80% and 7.5% DW, respectively, which was highly consistent with the results reported by Pagliuso et al. [5]. Importantly, duckweed contained an abundance of the EAAs recommended by the WHO (Table 2). This result indicated that natural duckweed was also a promising source of plant protein. Interestingly, people in some countries or regions, such as Thailand, Myanmar, Laos, and Southwest China, have long consumed duckweed as food [13]. Finally, the safety of duckweed as a food must be focused on. A previous study revealed that duckweed did not have any detectable adverse effects on human cells [31]. Zeinstra et al. [32] reported that no notable adverse reactions occurred after duckweed intake. Kaplan et al. [33] similarly concluded that duckweed was safe to eat. Taken together, these data show that natural duckweed is inexpensive, easily obtained, and capable of being a human food feedstock.

4.2. The Potential of Duckweed as a Novel Feedstock for Bioethanol Fermentation. In addition to being a human food feedstock, duckweed has been considered a bioenergy crop [7]. As listed in Table S1, duckweed biomass can be efficiently converted into bioethanol, and the duckweed-ethanol conversion rate is positively correlated with the starch content in duckweed. Thus, duckweed was cultivated under nutrient starvation to induce starch accumulation. The starch content and biomass yield were then evaluated. As shown in Figure 1(c), the starch content increased to 51.45% DW after 10 d of cultivation, which is similar to that in some grains [1]. Therefore, the duckweed starch content was high enough for bioethanol fermentation. Notably, 72.53 g/m^2 high-starch duckweed biomass can be harvested every 10 days (Figure 1(a)), thus leading to an annual yield of 26.11 t/ha/y, which was significantly higher than that of maize and wheat (Table 3). Finally, duckweed biomass was fermented into ethanol using SHF. The ethanol conversion rate ($Y_{E/G}$) reached 0.469 g/g glucose with 91.83% fermentation efficiency, revealing that in duckweed, starch can be efficiently converted into ethanol (Table 2). The efficiency of duckweed fermentation into ethanol (91.83%) was comparable to that of maize to ethanol and wheat to ethanol [28]. In addition, the ethanol yield ($Y_{E/B}$) reached 0.262 g/g, which, to our knowledge, is the highest ethanol yield based on duckweed fronds reported thus far (Table S1). Based on the biomass yield (26.11 t/ha/y), a potential duckweed-bioethanol volume of 8670 L/ha/y can be achieved, which is far higher than that of wheat-bioethanol (1,552 L/ha/y) and maize-bioethanol (2,585 L/ha/y) (Table 3). Consequently, high-starch duckweed can be used as a profitable feedstock for large-scale bioethanol production.

4.3. The Potential of Duckweed as an Antileukaemia Drug. Another important feature of duckweeds that has attracted scientific attention worldwide is their pharmaceutical effects. Duckweed has long been used as a folk medicine for the treatment of asthma, diabetes, rhinitis, and so forth in China and even in some European nations [12]. However, the anticancer effect of duckweed flavonoids has rarely been reported. Although Pagliuso et al. [34] speculated that duckweeds had anticancer effects based on their main flavonoids (apigenin, luteolin, and their derivatives), the real anticancer effects, especially antileukaemic effects, of duckweed flavonoids have not been systematically investigated. Therefore, the antileukaemic effects of duckweed were evaluated *in vitro* and *in vivo* for the first time. Acute leukaemia (AL) is the most common cancer among children and accounts for one-third of all childhood cancers [35]. Acute myeloid leukaemia (AML) accounts for only 15~20% of the cases but more than 30% of the deaths from AL [36]. In particular, the survival of refractory childhood AML patients is poor (<22%) [37]. Thus, it is essential to find more promising drugs for AML therapy. In this study, HL60 and Thp1 cells were chosen for further investigation. As shown in Figure 2, DFs showed a notable inhibitory effect on the growth of both types of cells. Based on the IC_{50} values, HL60 cells were more sensitive to DFs than Thp1. Thus, we can preliminarily conclude that DFs possess an antileukaemic effect. As shown in Figures 2(c)–2(e), DF induced apoptosis in both cell lines in a dose-dependent manner, indicating that DF effectively suppressed AML cell growth through apoptosis induction *in vitro*. To further confirm this conclusion, *in vivo* experiments were performed. The results indicated that DFs at 60 mg/kg inhibited tumour growth in xenograft mice. The DF inhibitory rates exceeded 45% for tumours originating from both cell lines and over 60% for those of the HL60 lineage. These results were in complete agreement with those of the *in vitro* experiments. Importantly, the body weights of the mice in the treatment group and control group exhibited no apparent difference, indicating that DFs can effectively inhibit AML *in vivo* without influencing body weight (Figure S2). Furthermore, DFs showed insignificant cytotoxicity on human or monkey normal cells (Figure S1). Collectively, the *in vitro* and *in vivo* experiments suggested that the use of DFs is a safe and effective strategy for AML therapy.

5. Conclusion

In this study, the nutritional value of natural duckweed was systematically evaluated for the first time. Considering the high yield of duckweed in the natural environment worldwide, it may be a great candidate for the global human food supply. Furthermore, the potential to produce bioethanol from duckweed was assessed. High-starch duckweed biomass was fermented into bioethanol, and the yield reached 0.262 g/g biomass, which was the highest ethanol yield using duckweed fronds reported thus far. Based on the duckweed biomass yield (26.11 t/ha/y), a potential duckweed-bioethanol yield of 8670 L/ha/y can be achieved, which is 4.6 and 2.4 times higher than that of wheat-bioethanol and maize-bioethanol, respectively. Duckweed, therefore, seems to be an excellent energy crop for bioethanol production. Finally, the antileukaemic effects of

duckweed flavonoids were evaluated for the first time. DFs significantly inhibited AML cells *in vitro* and *in vivo*, and the outcome supported DFs as effective agents for AML therapy. In conclusion, duckweed is a promising crop that could be helpful for humans to meet the challenges posed by food security, energy crises, and tumours. Certainly, the anticancer effect of DFs and the relevant molecular mechanism need to be investigated further.

Abbreviations

DF: Duckweed flavonoid
AML: Acute myeloid leukaemia
GR: Growth rate
SHF: Separate hydrolysis and fermentation
TAA: Total amino acid
EAA: Essential amino acid
NEAA: Nonessential amino acid
DW: Dry weight
FW: Fresh weight
AL: Acute leukaemia
RT: Room temperature.

Data Availability

The data used to support the findings of this study are included within the article and the supplementary information file(s).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Ling Guo, Jing Liu, and Qingyan Wang contributed equally to this work. Ling Guo conceptualized the study, performed the methodology, provided funding acquisition, performed supervision, performed investigation, and wrote the original draft. Jing Liu performed investigation and wrote the original draft. Qingyan Wang conceptualized the study, performed the methodology, and performed supervision. Yan Yang performed investigation and contributed to visualization. You Yang performed formal analysis and performed data curation. Qulian Guo performed supervision, performed the methodology, and provided resources. Hai Zhao conceptualized the study, performed the methodology, provided resources, and provided funding acquisition. Wenjun Liu conceptualized the study, performed the methodology, provided funding acquisition, and performed supervision. All authors wrote, reviewed, and edited the manuscript.

Acknowledgments

This work was funded by the Natural Science Foundation of Sichuan Province (2022NSFSC0723), the Opening Project of the State Key Laboratory of Non-Food Biomass and Enzyme Technology (2022-36-Z06-02), the Medical Science and Technology Project of Health Commission of Sichuan Province (no. 21PJ091), the Science and Technology Strategic Cooperation Project between Luzhou Municipal People's Government and Southwest Medical University (2021LZXNYD-J22), the Special Project of Science and Technology Research of Sichuan Administration of Traditional Chinese Medicine (2020JC0135), the Applied Basic Research Project of Southwest Medical University (2021ZKQN083, 2020ZRQNB029), the Doctoral Research Initiation Fund of the Affiliated Hospital of Southwest Medical University, Sichuan Science and Technology Program (2022YFS0622), the Biological Resources Program, Chinese Academy of Sciences (KFJ-BRP-008), the Innovation Academy for Seed Design, CAS, and the National Aquatic Biological Resource Center (NABRC).

Supplementary Materials

Table S1: the data of different technologies and ethanol yields from different duckweed varieties in the previous studies and this study. Figure S1: the cytotoxicity of duckweed on human or monkey normal cells. Figure S2: the data of mouse body weight after DFs treatment. The results indicated that 60 mg/kg DFs had no significant effect on mouse body weight. (*Supplementary Materials*)

References

[1] L. Guo, Y. Jin, Y. Xiao et al., "Energy-efficient and environmentally friendly production of starch-rich duckweed biomass using nitrogen-limited cultivation," *Journal of Cleaner Production*, vol. 251, Article ID 119726, 2020.

[2] K.-J. Appenroth, K. S. Sree, V. Bohm et al., "Nutritional value of duckweeds (Lemnaceae) as human food," *Food Chemistry*, vol. 217, pp. 266–273, 2017.

[3] M. F. A. de Beukelaar, G. G. Zeinstra, J. J. Mes, and A. R. H. Fischer, "Duckweed as human food. The influence of meal context and information on duckweed acceptability of Dutch consumers," *Food Quality and Preference*, vol. 71, pp. 76–86, 2019.

[4] Z. Hu, Y. Fang, Z. Yi et al., "Determining the nutritional value and antioxidant capacity of duckweed (Wolffia arrhiza) under artificial conditions," *LWT - Food Science and Technology*, vol. 153, Article ID 112477, 2022.

[5] D. Pagliuso, A. Grandis, J. S. Fortirer, P. Camargo, E. I. S. Floh, and M. S. Buckeridge, "Duckweeds as promising food feedstocks globally," *Agronomy*, vol. 12, no. 4, p. 796, 2022.

[6] J. Xu, Y. Shen, Y. Zheng et al., "Duckweed (Lemnaceae) for potentially nutritious human food: a review," *Food Reviews International*, vol. 1-15, pp. 1–15, 2021.

[7] C. Chang, J. L. Bowman, and E. M. Meyerowitz, "Field guide to plant model systems," *Cell*, vol. 167, no. 2, pp. 325–339, 2016.

[8] K.-J. Appenroth, P. Ziegler, and K. S. Sree, "Accumulation of starch in duckweeds (Lemnaceae), potential energy plants," *Physiology and Molecular Biology of Plants*, vol. 27, no. 11, pp. 2621–2633, 2021.

[9] Y. Yang, Y. Chen, Y. Jin et al., "Highly efficient fermentation of glycerol and 1,3-propanediol using a novel starch as feedstock," *Food Bioscience*, vol. 46, Article ID 101521, 2022.

[10] N. Shen, H. Zhang, Y. Qin et al., "Efficient production of succinic acid from duckweed (*Landoltia punctata*)

hydrolysate by *Actinobacillus succinogenes* GXAS137," *Bioresource Technology*, vol. 250, pp. 35–42, 2018.

[11] X. Ge, N. Zhang, G. C. Phillips, and J. Xu, "Growing Lemna minor in agricultural wastewater and converting the duckweed biomass to ethanol," *Bioresource Technology*, vol. 124, no. 337, pp. 485–488, 2012.

[12] Y. V. Bolotova, "Aquatic plants of the Far East of Russia: a review on their use in medicine, pharmacological activity," *Bangladesh Journal of Medical Science*, vol. 14, no. 1, pp. 9–13, 2015.

[13] G. Baek, M. Saeed, and H.-K. Choi, "Duckweeds: their utilization, metabolites and cultivation," *Applied Biological Chemistry*, vol. 64, no. 1, p. 73, 2021.

[14] Y. Yin, C. Yu, L. Yu et al., "The influence of light intensity and photoperiod on duckweed biomass and starch accumulation for bioethanol production," *Bioresource Technology*, vol. 187, pp. 84–90, 2015.

[15] S. Gao, Y. Kong, Y. Lv, B. Cao, Z. Chen, and K. Xu, "Effect of different LED light quality combination on the content of vitamin C, soluble sugar, organic acids, amino acids, antioxidant capacity and mineral elements in green onion (Allium fistulosum L.)," *Food Research International*, vol. 156, Article ID 111329, 2022.

[16] B. Tsolmon, Y. Fang, T. Yang et al., "Structural identification and UPLC-ESI-QTOF-MS 2 analysis of flavonoids in the aquatic plant Landoltia punctata and their in vitro and in vivo antioxidant activities," *Food Chemistry*, vol. 343, no. 1, Article ID 128392, 2021.

[17] X. Qiao, W. N. He, C. Xiang et al., "Qualitative and quantitative analyses of flavonoids in Spirodela polyrrhiza by high-performance liquid chromatography coupled with mass spectrometry," *Phytochemical Analysis*, vol. 22, no. 6, pp. 475–483, 2011.

[18] D. Perez-Rea, C. Rojas, S. Carballo, W. Aguilar, B. Bergenståhl, and L. Nilsson, "Enzymatic hydrolysis of Canna indica , Manihot esculenta and Xanthosoma sagittifolium native starches below the gelatinization temperature," *Starch - Stärke*, vol. 65, no. 1-2, pp. 151–161, 2013.

[19] C. N. Economou, A. Makri, G. Aggelis, S. Pavlou, and D. V. Vayenas, "Semi-solid state fermentation of sweet sorghum for the biotechnological production of single cell oil," *Bioresource Technology*, vol. 101, no. 4, pp. 1385–1388, 2010.

[20] Y. H. Huang, Y. L. Jin, Y. Fang et al., "Simultaneous saccharification and fermentation (SSF) of non-starch polysaccharides and starch from fresh tuber of *Canna edulis ker* at a high solid content for ethanol production," *Biomass and Bioenergy*, vol. 52, pp. 8–14, 2013.

[21] J. Medić, L. J. Abendroth, R. W. Elmore, and J. L. Jane, "Effect of planting date on maize starch structure, properties, and ethanol production," *Starch Staerke*, vol. 68, no. 5-6, pp. 476–487, 2016.

[22] J. Liu, L. Zhang, L. Guo et al., "Novel bioactive hybrid Celecoxib-HDAC Inhibitor, induces apoptosis in human acute lymphoblastic leukemia cells," *Bioorganic and Medicinal Chemistry*, vol. 75, Article ID 117085, 2022.

[23] L. Guo, J. Liu, Y. Yang et al., "Purple sweet potato anthocyanins elicit calcium overload-induced cell death by inhibiting the calcium-binding protein S100A4 in acute lymphoblastic leukemia," *Food Bioscience*, vol. 42, Article ID 101214, 2021.

[24] L. Guo, Y. Jin, Y. Yang et al., "Calcicoptosis induced by purple sweet potato anthocyanins through the nonosmotic regulation of the NFAT5/S100A4-S100A9 pathway in acute lymphoblastic leukemia," *Chemistry and Biodiversity*, vol. 19, no. 9, Article ID e202200447, 2022.

[25] I. F. A. D. Fao, W. F. P. Unicef, and Who, "The state of food security and nutrition in the world 2020," in *Transforming Food Systems for Affordable Healthy Diets)*FAO, Rome, 2020.

[26] Z. Zhou, K. Robards, S. Helliwell, and C. Blanchard, "Composition and functional properties of rice," *International Journal of Food Science and Technology*, vol. 37, no. 8, pp. 849–868, 2002.

[27] T. Wang, M. Wang, S. Hu et al., "Genetic basis of maize kernel starch content revealed by high-density single nucleotide polymorphism markers in a recombinant inbred line population," *BMC Plant Biology*, vol. 15, no. 1, p. 288, 2015.

[28] J. M. Adams, J. A. Gallagher, and I. S. Donnison, "Fermentation study on Saccharina latissima for bioethanol production considering variable pre-treatments," *Journal of Applied Phycology*, vol. 21, no. 5, pp. 569–574, 2009.

[29] S. R. Bush, B. Belton, D. C. Little, and M. S. Islam, "Emerging trends in aquaculture value chain research," *Aquaculture*, vol. 498, pp. 428–434, 2019.

[30] K.-J. Appenroth, K. S. Sree, M. Bog et al., "Nutritional value of the duckweed species of the genus Wolffia (lemnaceae) as human food," *Frontiers of Chemistry*, vol. 6, p. 483, 2018.

[31] K. S. Sree, H.-M. Dahse, J. N. Chandran, B. Schneider, G. Jahreis, and K. J. Appenroth, "Duckweed for human nutrition: No cytotoxic and No anti-proliferative effects on human cell lines," *Plant Foods for Human Nutrition*, vol. 74, no. 2, pp. 223-224, 2019.

[32] G. G. Zeinstra, D. Somhorst, E. Oosterink et al., "Postprandial amino acid, glucose and insulin responses among healthy adults after a single intake of Lemna minor in comparison with green peas: a randomised trial," *Journal of nutritional science*, vol. 8, p. e28, 2019.

[33] A. Kaplan, H. Zelicha, G. Tsaban et al., "Protein bioavailability of Wolffia globosa duckweed, a novel aquatic plant - a randomized controlled trial," *Clinical Nutrition*, vol. 38, no. 6, pp. 2576–2582, 2019.

[34] D. Pagliuso, C. E. Palacios Jara, A. Grandis, E. Lam, M. J. Pena Ferreira, and M. S. Buckeridge, "Flavonoids from duckweeds: potential applications in the human diet," *RSC Advances*, vol. 10, no. 73, pp. 44981–44988, 2020.

[35] R. Masetti, E. Muratore, D. Leardini et al., "Gut microbiome in pediatric acute leukemia: from predisposition to cure," *Blood Advances*, vol. 5, no. 22, pp. 4619–4629, 2021.

[36] J. E. Rubnitz, B. Gibson, and F. O. Smith, "Acute myeloid leukemia," *Hematology-Oncology Clinics of North America*, vol. 24, no. 1, pp. 35–63, 2010.

[37] P. Merli, "Treating second-relapsed/refractory first-relapsed childhood acute myeloid leukaemia: successful salvage rather than palliation?" *British Journal of Haematology*, vol. 197, no. 6, pp. 651-652, 2022.