

Health oil preparation from gardenia seeds by aqueous enzymatic extraction combined with puffing pre-treatment and its properties analysis

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Abstract

Gardenia jasminoides Ellis, a typical representative for “homology of medicine and food”, can be used to produce gardenia yellow and edible oil. Here, aqueous enzymatic extraction (AEE) combined with puffing pre-treatment was explored to prepare oil from gardenia seeds. Free oil yield (FOY) by AEE was evaluated in term of selection of enzyme, enzyme combination, as well as puffing ways and conditions. Results suggested that seeds taken as raw materials were better than whole fruit, sequential hydrolysis was superior to single enzymatic hydrolysis. Both wet-heating puffing at 90°C and dry-heating puffing at 1.0MPa facilitated the release of free oil by AEE, while FOY produced the highest oil yields of 21.8% and 23.2% within 3h, and 58.3%, 68.4% higher than that of un-puffed group, active crocin and geniposide were also completely released. The FOY obtained in this study was far higher than pressing method (10.44%), and close to solvent extraction (25.45%). Also, microstructure analysis indicated that gardenia seeds expanded by dry-heating puffing (1.0MPa) had larger and rougher surface and porous structure than other groups. Moreover, there was no significant difference between oils prepared by various methods in term of nutritional and safety characteristics. AEE coupled with puffing pre-treatment developed is an eco-friendly extraction technology with high efficiency that can be employed to oil preparation enriched with active crocin and geniposide from gardenia seeds.

1. Introduction

Gardenia jasminoides Ellis (GjE), an evergreen shrub widely distributed in Asian countries, is always used for making tea, cooking porridge and coloring dishes, whose ripe fruit attain a reddish yellow color (Li et al., 2020; Wang et al., 2022). The desiccative ripe fruits of GjE have been applied in China for centuries as both a food and medicinal substance (Chen et al., 2020). Up to present, several useful chemical constituents have been isolated from GjE and characterized, mainly including geniposide, geniposidic acid, genipin, crocins and their derivatives. Various physiological function has been reported, including anti-inflammatory, antioxidant properties, anti-hypertension, anti-hyperglycemia, anti-cancer, anti-hyperlipidemia, neuroprotection and hepatoprotection. Furthermore, the crude fat content takes up nearly 20% of the dried fruit, and its oil level is equivalent to that of soybean, which can be applied to oil production (Chyau et al., 2022; Yin and Liu, 2018). According to previous study, gardenia oil containing plenty of Unsaturated fatty acids, particularly palmitic acid and linoleic acid, which exhibits pharmacological effects on regulating blood pressure, body fat metabolism, and reducing serum cholesterol (Cai et al., 2015). Tao et al. (2014) stated that the gardenia oil may contain effective constituents to be used for depression therapy. Thus, gardenia oil can be regarded as a kind of oil with excel quality and health benefits. Nowadays, plant oils (especially nut oils) have been applied by the food industry to develop nutraceutical products due to their healthy fatty acid composition, richness in sterols, fat-soluble vitamins, phenolic compounds and distinct flavors (Ferreira et al., 2022). However, a large amount of gardenia meals was discarded as waste after extraction of gardenia yellow pigment. Hence, preparation of valued oil from discarded gardenia meals is meaningful, not only for increase in the value of GjE, but also for meeting the growing market demand of natural healthy plant oils.

With regard to extraction of oil from gardenia meals, cold pressing extraction (CPE), solvent extraction (SE), supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE) were commonly used methods, each has its advantages and limitations (Xiao et al., 2017). CP can greatly maintain the unique flavor of oils, but low yield, high labor intensity, resulted in higher produce cost (Xu et al., 2021). Three conventional extractions including cold pressing, petroleum ether extraction and ultrasound-assisted extraction (UAE) methods were compared by Cai et al (2015). The oil extraction yields of 8.59%, 7.4%, 5.6% and 10.89% were obtained for UAE, petroleum ether extraction, cold pressing and Soxhlet extraction, indicated the high extraction efficiency of UAE. Nevertheless, SE is generally applied to extraction on large scale. Extensive refine resulted loss of active compounds and solvent residual limited its use in small oilseeds with high value. He et al. (2010) and Tao et al. (2014) explored supercritical carbon dioxide (SC-CO₂) extraction of oil from whole gardenia fruits, extraction pressure, temperature and CO₂ flow rate were optimized and the highest oil yields of 9.77% and 11.48% were obtained. SFE is a realistic green extraction method which omitted the use of organic solvent, the extracted oil is natural and safe. Meanwhile, high investment costs and small extraction capacity may be the biggest obstacle for widely use. (Jha and Sit, 2022). Recently, AEE as an emerging plant oil extraction technology, showed more and more advantages compared with conventional extraction methods. It is eco-friendly, mild reaction conditions, cost-saving and reserves health benefit of plant oils. Its application covered sunflower seeds, camellia seeds, rice bran, peanuts, *Cinnamomum camphora* seeds (De Aquino et al., 2022; Jiang et al., 2010; Meng et al., 2018; Wei et al., 2022; Xu et al., 2021). Gardenia fruit composition was unique compared with these oilseeds. While few literature was found for gardenia oil extraction by AEE, hence it is necessary to perform oil preparation form gardenia fruits by using AEE.

For AEE process in term of oil preparation, disruption degree of the oleaginous materials cell walls by enzyme is important factor in term of oil yield and extraction efficiency. Before hydrolysis, puffing pre-treatment on gardenia seeds may be helpful for improving extraction efficiency. Puffing is the process of suddenly decline pressure at high temperature and pressure, thus resulting in a porous texture of food matrix (Kim et al., 2018). High pressure can be achieved by heating vaporization, superheated steam, mechanical extrusion, compressed air treatment in a closed cavity. However, specific puffing effects of each treatment and their impact on yield and quality of oil by AEE are not clear.

In this work, gardenia seeds are first pre-treated by various puffing methods, then 60% isopropanol is used to recover bioactive crocin and geniposide which will decrease their involvement into emulsion. The obtained gardenia meals are used to prepare oil by AEE, optimized in term

of puffing conditions and hydrolysis parameters. Furthermore, we evaluate the microstructure, specific surface area, pore volume, and pore size of puffed gardenia seeds to investigate the degree of cell wall damage. The gardenia seed oil is evaluated in comparison with oil by traditional methods with respect to nutritional composition and quality.

2. Materials And Methods

2.1. Plant materials and chemicals

Gardenia jasminoides Ellis fruits were purchased from the local market (Hangzhou, China). Cellulase (EC 3.2.1.4 from *Aspergillus niger*, enzyme activity, 10000 U/g) and pectinase (EC 3.2.1.15 from *Aspergillus niger*, enzyme activity, 30000 U/g) were obtained from Aladdin Bio-Chem Technology, Shanghai, China. Alcalase (alkaline serine endopeptidase from *Bacillus licheniformis*, enzyme activity, 280800 U/g) was purchased from Novozyme, TianJin, China. Geniposide ($\geq 98\%$) and Crocin (for microscopy) were obtained from Sigma-Aldrich Co. (Shanghai, China). All other chemicals and solvents used were of analytical and chromatographic grade.

2.2. Nutritional composition

The moisture, protein (Kjeldahl method), fat (Soxhlet extraction) of the whole and the dehulled gardenia fruits were determined according to the AOAC (1990) methods. Carbohydrate was analyzed according to method reported by Rehman et al. (2001). The pectin and cellulose were determined by colorimetric hydroxyl-phenyl-phenol method and anthrone colorimetry method as described by Wang et al. (2021).

2.3. Puffing pre-treatment

2.3.1 Dry-heating puffing (DP)

The dehulled samples were first adjusted the moisture to 12%, then placed into puffing tank (B-H, Hangzhou Chengyuan Trading Co., LTD, Hangzhou). The puffing tank is heated by charcoal fire while rotating and stirring until the pressure in the tank rises to 0.5, 0.6, 0.7, 0.8, 1.0 MPa, and then the pressure is instantly released to atmospheric pressure. The puffing process finishes.

2.3.2 Wet-heating puffing (WP)

The dehulled samples were soaked in deionized water to rehydrate to 12%, and then sent it into the puffing tank. The puffing tank was pressurized by air compressor, and steam was injected into the puffing tank. When the temperature rose to 80, 90, 100, 110, 120 °C, the pressure was adjusted to 1.0 MPa and kept for 5min. Vacuum valve connecting vacuum tank and puffing tank was opened, the puffing pre-treatment was completed at this moment. Then the puffed sample was dried at 80°C for 2 ~ 3h until the desired moisture content (4 ~ 5%) was reached (Yifu Zhang et al., 2021).

2.4. Aqueous enzymatic extraction process

Dried gardenia seeds were pulverized by using a Moulinex AR1044 grinder and screened through a 40-mesh sieve. Before hydrolysis, the gardenia powder was first extracted with methanol, ethanol or isopropanol at a ratio of 1:5 (w/v) assisted by ultrasound (100W for 30 min) to remove bioactivities, which may enhance the following emulsion. The residual pomace was mixed with distilled water at a ratio of 1:7 (w/v), and subjected to heat treatment of 90°C for 10 min. When the mixture was cooled down to desired temperature, various enzyme was added and enzymatic hydrolysis processes was performed at optimal condition of each enzyme. The flowchart of the AEE process is presented in Fig. 1.

2.4.1. Single enzyme hydrolysis.

The single enzyme was added after the suspension pH was adjusted to the supplier recommended pH for each enzyme (using 0.1M NaOH or HCl aqueous solutions). The enzyme hydrolysis was proceeding under constant horizontal shaking in a rotary shaker at optimal temperature. After the reaction, the mixture was heated to 100°C for 10 min to deactivated enzyme. The enzyme hydrolysis solution was centrifuged at 8000 × g for 20 min at 4°C. The upper free oil was extracted three times using 15ml of n-hexane and hexane was removed by rotary evaporation, free oil yield was calculated (Díaz-Suárez et al., 2021).

2.4.2. Sequential hydrolysis by multiple enzymes

After the hydrolysis process of first enzyme, the free oil was recovered or not, then the mixture was adjusted to the optimal pH and temperature for another enzyme, the second stage enzymatic hydrolysis by another enzyme was proceeded. Last, the free oil was recovered and calculated the free oil yield. The optimal conditions of solid to liquid ratio (w/w), pH, temperature and extraction time of the hydrolysis were obtained through single-factor experiments. Free oil yield (FOY) was calculated as the mass of free oil extracted (g) divided by the mass of the initially processed samples (g) and then multiplied by 100.

2.5. Cold pressing extraction and Soxhlet extraction process

Cold pressing extraction (CPE) and solvent extraction (SE) process were proceeded according to the previous publication with minor modifications (Polmann et al., 2019; Tang et al., 2021). Gardenia fruits was placed into a CA 59G KOMET press (IBG Monforts GmbH & Co., Germany) at the speed of 25 r/min, the whole process is allowed to proceed under 70°C. The oil was recovered by centrifugation (4500 r/min, 10 min). For Soxhlet extraction, 5g gardenia powder and 150 mL petroleum ether were added into a Soxhlet apparatus, extraction was performed under 80°C for 8 h. After extraction, the extract was collected and evaporated under reduced pressure with a rotary evaporator Model RE-2000A (Yarong Biochemical Instrument Factory, Shanghai, China) at 45 °C. The remained oil was dried under nitrogen flow, weight and calculate the oil yield. The oil obtained was kept at 4°C until use.

2.6. Scanning electron micrographs (SEM)

The morphology of raw gardenia seed powder or puffed gardenia powder was investigated with the field emission scanning electron microscope (SEM, Zeiss Gemini 500, Germany). Firstly, samples were fixed on the aluminum plate with double-sided carbon tape, then sprayed with a thin layer of gold-palladium at 5 kV. Finally, samples were observed at an accelerating voltage of 5 kV with a magnification factor of 2500 × (Hu et al., 2019).

2.7. Brunauer-Emmett-Teller (BET) specific surface area, pore volume and pore size

The BET specific surface area, pore volume and pore size distribution of puffed gardenia seeds were determined by an automatic rapid surface area and microporous analyzer (ASAP 2010, Micromeritics, USA). N₂ was selected as the adsorption and desorption gas. Puffed gardenia seeds were placed in the measuring cell to degas water by heating at 150°C for 12h at least. Afterwards, the measuring cell was placed in an insulated tank filled with liquid nitrogen to keep the sample at -196°C throughout the measurement. The specific surface area was determined by the BET isotherm multipoint method in the relative pressure (P/P₀) range of 0–1.0. Pore volume and pore size distribution were obtained from the desorption isotherms by Barrett–Joyner–Halenda (BJH) method (Beluns et al., 2021).

2.8. HPLC-MS/MS analysis for bioactive compounds from gardenia seeds.

The geniposide and crocin in raw and puffed gardenia seeds as well as in hydrolysate by AEE were determined by a high performance liquid chromatography (Waters E2695) coupled with a Agilent SB-C18 (4.6 × 250mm, 5μm) column. Acetonitrile: water = 50:50 was used as mobile phase for isometric elution. The flow rate was 1.0 mL/min, and the injection volume was 10μL. Peak area at 238 nm and 440 nm were measured respectively for quantitation of geniposide and crocin according to each calibration.

HPLC-MS/MS analysis conditions were listed as follows, multiple reaction monitoring mode was used to monitor parent and fragment ions of analytes. The ion source temperature, 500°C; the nebulizer gases were maintained at 0.345MPa (Zhang et al., 2023).

2.9. Evaluation of gardenia fruit oil properties

2.9.1. Physicochemical features

Samples were characterized according to the official methods of AOCS (1997) as follows: acid value (Cd 3d-63), peroxide value (Cd 8–53), iodine value (Cd 1d-92). Saponification value was determined based on the protocol issued by the Ministry of Agriculture (China) of GB/T 5534 – 2008 (Gai et al., 2013).

2.9.2. Fatty acid composition analysis

The fatty acid composition was determined according to the IUPAC method 2.301, after fatty acid methyl esters (FAMES) of gardenia seed oil (Nguyen et al., 2020). GC-MS analysis was performed on a Thermo 1300 gas chromatography/mass spectrometer (Varian, Santa Clara, CA, USA), equipped with an DB-Wax silica capillary column (60m×0.32mm×0.25μ). The mass spectrometer was operated in positive ion mode with an ionization energy of 70 eV. The GC–MS analysis conditions were as follows: initial capillary operated temperature of 90 °C (held for 5 min), raised to 200°C at 10°C/min (kept for 10 min), increased to 220°C at 0.5°C/min (kept for 5 min), raised to 240°C at 5°C/min, and maintained for 10min. The temperatures for injector, detector and ion source were 230, 250 and 230°C, respectively. Oxygen-free nitrogen was used as carrier gas with a split ratio of 1:40. Mass units were monitored from m/z 40 to 500. The components were designated by comparison to the NIST library mass spectra. The quantity of fatty acids was calculated by the peak area normalization law and represented as the relative percent of each fatty acid to the total fatty acids (Liu et al., 2019).

2.10. Statistical analysis

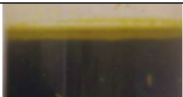
All the samples (each sample with replicates data) were analyzed and the average value for each sample was performed using the tool available in OriginLab 2022b (OriginLab, MA, USA). The results are expressed as the mean ± standard error. The difference between data groups were tested by one way ANOVA and Duncan's multiple range tests (SPSS for Windows software release 18; SPSS Inc., New York, USA). The chosen level was considered significant when $p < 0.05$.

3. Results And Discussion

3.1. Nutritional composition analysis for gardenia fruits and gardenia seeds

The proximate composition of gardenia fruits including geniposide, and crocin was determined (Table 1). The whole gardenia fruit contained moisture of $5.53 \pm 0.19\%$, crude fat of $19.99 \pm 0.92\%$, crude protein of $9.97 \pm 0.58\%$, cellulose of $14.11 \pm 0.57\%$, pectin of $14.29 \pm 0.57\%$, geniposide of $6.32 \pm 0.17\%$ and crocin of $9.56 \pm 0.13\%$. The nutritional composition was similar with those reported by Chyau et al. (2022). After the removal of shell, the contents of moisture, fat, protein, geniposide and crocin in gardenia seed were increased to $6.04 \pm 0.14\%$, $24.92 \pm 0.06\%$, $11.79 \pm 0.16\%$, $7.68 \pm 0.06\%$, and $10.45 \pm 0.07\%$, while cellulose and pectin decreased to $11.93 \pm 0.29\%$ and $11.74 \pm 0.24\%$, indicating that the lipids, geniposide and crocin tend to be enriched in the gardenia seeds, the peel is abundant of cellulose and pectin. It was worth noting that the crude fat content took up nearly 25% of the gardenia seeds, equivalent to the oil content of soybean, which showed tremendous potential to be used as a valuable oil crop. The full gardenia fruit and dehulled gardenia seed were tested to hydrolyze with cellulase with a pH of 6.0 at 50°C for 1.5h. The pictures for products obtained was shown in Table 1. Apparently, severe emulsification was found for the full gardenia hydrolysate so as to no free oil was extracted. This may due to too much polysaccharide (especially for pectin) set free from peel involved into the emulsion. For gardenia seeds hydrolysate, clear oil-water interface was found, and free oil yield of $9.07 \pm 0.22\%$ was obtained. Based on these finding, gardenia seeds were used to prepare oil by AEE in subsequent experiments.

Table 1. Proximate composition, geniposide and crocin content in the gardenia fruit.

Samples	Moisture (%)	Fat (%)	Protein (%)	Cellulose (%)	Pectin (%)	Geniposide (%)	Crocin (%)	Visual appearance of oil obtained
Whole fruit	5.53±0.19	19.99±0.92	9.97±0.58	14.11±0.8	14.29±0.57	6.32±0.17	9.56±0.13	
Dehulled fruit (seed)	6.04±0.14	24.92±0.06	11.79±0.16	11.93±0.29	11.74±0.24	7.68±0.06	10.45±0.07	

3.2 Aqueous enzymatic extraction for gardenia seed oil.

3.2.1. Bioactive compounds recovery by solvent extraction and its impact on free oil yield by AEE

Geniposide and crocin are the major bioactive components for gardenia fruit, which have widely pharmacological properties such as hepatoprotective, choleric, neuroprotective, anti-inflammatory, antioxidant, antitumor, anti-apoptotic and anti-diabetic activities (Tian et al., 2022). Hence, before AEE process, recovering geniposide and crocin by solvent was essential for value-added full utilization for gardenia crop. The solvent used should be polar enough to extract geniposide and crocin, but against hydrophobic oil. So, methanol, ethanol and isopropanol solution at different concentration were used to extract geniposide and crocin, the residual meal were hydrolyzed by cellulase to prepare gardenia seed oil. Yields for geniposide and crocin and FOY were shown in Fig. 2. Result indicated that 60% ethanol extraction resulted in the optimum crocin yield of $6.42 \pm 0.17\%$, while 60% isopropanol gave the best geniposide yield of $6.18 \pm 0.34\%$ and the highest free oil yield of $11.46 \pm 0.30\%$. The higher removal for amphiphile geniposide by 60% isopropanol may decreased its involvement into emulsion, produced a higher FOY. Thus, the pre-recover of geniposide and crocin by 60% isopropanol was deserved before performing AEE for gardenia seeds.

3.2.2. Gardenia seed oil preparation by various aqueous enzymatic extraction processes

In the oil crop cells, oils are usually present in lipid cells surrounded by components such as lignin, pectin, hemicellulose, cellulose and protein in form of a complex that binds other macromolecules (Li et al., 2011). In view of chemical composition characteristic of high cellulase, pectin and protein level for gardenia seeds, cellulase (Cel), pectinase (Pec), protease (Pro) or their combination was used to disrupt the cell wall and lipid complex. Under each optimal conditions including pH, temperature, and enzyme load, AEE processes were performed. The FOY and geniposide, crocin level in hydrolysate were determined and compared, as shown in Table 2. For single enzymatic hydrolysis, no matter for Cel, Pec or Pro, FOYs were all between 12.42%~12.58%, no significant difference was found among groups. However, the optimal hydrolysis time for Cel is the shortest with value of 1.5h compared with 2.5 h for Pec and 4h for Pro.

Table 2
Free oil yield and its geniposide and crocin content under different enzymatic hydrolysis process and different pre-treatment.

Hydrolysis process		Single enzyme hydrolysis		Two-stage enzyme hydrolysis								
				Without free oil removal at reaction interval				With oil removal at reaction interval				
Combinative enzymes		Cel	Pec	Pro	Cel + Pro	Pec + Pro	Cel + Pec	Cel + Pec + Pro	Cel + Cel	Pec + Pec	Pec + Cel	Cel + Pec
Enzyme concentrations (%)		0.8	0.6	0.8	0.8 + 0.6	0.6 + 0.8	0.8 + 0.6	0.8 + 0.6 + 0.6	0.8 + 0.8	0.6 + 0.6	0.6 + 0.8	0.8 + 0.6
pH		6	3.5	8.5	6→8.5	3.5→8.5	6→3.5	6→3.5→8.5	6	3.5	3.5→6	6→3.5
Incubation time (h)		1.5	2.5	4	1.5 + 4	2.5 + 4	1.5 + 3	1.5 + 2.5	1.5 + 1.5	2.5 + 2.5	2.5 + 1.5	1.5 + 2.5
Incubation temperature (°C)		50	50	60	50→60	50→60	50	50→50→60	50	50	50	50
Non-pre-treatment	FOY (%)	12.45 ± 0.32 _a	12.42 ± 0.62 _a	12.58 ± 0.40 _{ab}	14.96 ± 0.58 _{de}	14.52 ± 0.36 _{cde}	13.03 ± 0.27 _e	15.32 ± 0.31 _f	13.75 ± 0.43 _{bcde}	13.50 ± 0.17 _{abcd}	13.36 ± 0.75 _{abc}	13.46 ± 0.60 _{abcd}
	Geniposide (%)	0.48 ± 0.07 _{ab}	0.61 ± 0.03 _a	0.50 ± 0.12 _{ab}	0.37 ± 0.09 _{ab}	0.44 ± 0.07 _{ab}	0.39 ± 0.06 _{ab}	0.27 ± 0.09 _b	0.41 ± 0.05 _{ab}	0.48 ± 0.07 _{ab}	0.48 ± 0.02 _{ab}	0.45 ± 0.03 _{ab}
	Crocin (%)	1.07 ± 0.25 _{ac}	1.52 ± 0.04 _b	1.34 ± 0.14 _{bc}	0.83 ± 0.12 _{ad}	1.06 ± 0.08 _{ac}	0.94 ± 0.12 _{acd}	0.66 ± 0.05 _d	1.08 ± 0.12 _{ac}	1.22 ± 0.03 _{abc}	1.24 ± 0.13 _{abc}	1.01 ± 0.11 _{ac}
DP pre-treatment (0.6MPa)	FOY (%)	16.81 ± 0.54 _a	16.51 ± 0.25 _a	14.46 ± 0.36 _b	16.82 ± 0.61 _a	16.46 ± 0.32 _a	16.97 ± 0.41 _a	17.85 ± 0.54 _c	19.65 ± 0.34 _d	19.31 ± 0.59 _d	19.39 ± 0.28 _d	19.42 ± 0.56 _d
	Geniposide (%)	0.43 ± 0.07 _{ab}	0.58 ± 0.04 _b	0.55 ± 0.10 _b	0.33 ± 0.06 _a	0.41 ± 0.02 _{ab}	0.39 ± 0.12 _{ab}	0.20 ± 0.03 _c	0.39 ± 0.09 _a	0.41 ± 0.05 _{ab}	0.42 ± 0.06 _{ab}	0.41 ± 0.03 _{ab}
	Crocin (%)	0.70 ± 0.02 _a	1.07 ± 0.05 _b	0.97 ± 0.03 _b	0.61 ± 0.02 _{ac}	0.71 ± 0.03 _a	0.43 ± 0.10 _c	0.21 ± 0.01 _d	0.65 ± 0.06 _a	1.00 ± 0.01 _b	0.98 ± 0.15 _b	0.61 ± 0.03 _{ac}
WP pre-treatment (100°C)	FOY (%)	15.64 ± 0.71 _{ab}	15.44 ± 0.50 _{ab}	14.08 ± 0.56 _a	15.46 ± 0.43 _{ab}	15.31 ± 0.51 _{ab}	15.39 ± 0.39 _{ab}	16.69 ± 0.31 _{bc}	18.23 ± 0.42 _c	17.86 ± 0.43 _c	18.09 ± 0.5 _c	18.30 ± 0.65 _c
	Geniposide (%)	0.46 ± 0.05 _{ab}	0.55 ± 0.13 _a	0.57 ± 0.02 _a	0.40 ± 0.03 _{ab}	0.42 ± 0.13 _{ab}	0.39 ± 0.08 _{ab}	0.24 ± 0.06 _b	0.35 ± 0.05 _{ab}	0.47 ± 0.01 _{ab}	0.39 ± 0.11 _{ab}	0.42 ± 0.04 _{ab}
	Crocin (%)	0.83 ± 0.03 _a	1.39 ± 0.07 _b	1.23 ± 0.01 _b	0.63 ± 0.05 _c	0.97 ± 0.02 _{ad}	0.63 ± 0.12 _c	0.50 ± 0.03 _c	0.80 ± 0.12 _a	1.22 ± 0.03 _b	1.05 ± 0.11 _d	0.83 ± 0.07 _a
Values were the mean ± SD of three gardenia seed oils which was analyzed in triplicate												
Significant differences between the two processes in the same column without the same letter (<i>p</i> > 0.05)												

To enhance the oil preparation performance, sequence hydrolysis by complex enzymes was performed. Data suggested that Cel + Pec increase the FOY to 13.03%, but did not reach to as we expected. Pec + Pro and Cel + Pro further improve the FOY to 14.52% and 14.96% within 6.5 and 5.5h, respectively. Moreover, Cel + Pec + Pro produced the highest FOY of 15.32%. This accords those Liu et al. (2019) and Wei et al. (2022) reported when prepared *S. mukorossi* seed kernels oil and *Cinnamomum camphora* seeds oil by using AEE method. Commonly, the choice of enzyme depends on the cell composition of the oil-bearing material. Phytochemically, cellulose, hemicellulose and pectin are the three main components that constitute the primary wall, secondary wall and middle lamella of the cell wall of oil crops (Hu et al., 2020). In addition, the protein network of the lipid-based membranes surrounding the lipid corpuscles need to be effectively dissolved and hydrolyzed to release oil from the cell. Nevertheless, complex hydrolysis process and long hydrolysis time as 8h for Cel + Pec + Pro group were not desired industrially.

During combined hydrolysis, oil product removal between stage-hydrolysis did not further improve the FOY for Cel + Cel, Pec + Pec, Pec + Cel and Cel + Pec combinations, with values between 13.36–13.75%.

3.3. Oil preparation by AEE from puffed gardenia seeds under various conditions

In view of high crude fat content of 25% for gardenia seeds, however only 15% oil was extracted. Even after eliminating product inhibition, sequential enzymatic hydrolysis still could not reach a satisfied FOY. These indicated that oilseed structure was not destroyed completely by AEE process resulted in inadequate release for bound oil. Puffing was reported to be an effective method to create a porous structure so as to expand the apparent volume of oilseed. Before AEE, the dry-heat puffing (0.6 MPa) and wet-heat puffing (100 °C) were performed for gardenia seed, and the FOYs were compared with those from unpopped seeds and listed in Table 2. The data suggested that the effect of puffing is obvious, and dry-heat puffing is superior to wet-heat puffing in term of oil release by AEE. For single enzymatic hydrolysis process, the FOYs increased from about 12% for control to 14.08%~15.64% for wet-heat puffing at 100°C and 14.46%~16.81% for dry-heating puffing at 0.6 MPa. While for sequential enzymatic hydrolysis process, the FOYs were further increased to 15.31%~18.30% and 16.46%~19.65%, respectively. What should be particular noted is when dry-heating gardenia seeds puffed at 0.6 MPa was hydrolyzed by Cel + Cel produced the highest FOY of 19.65% within 3h. Thus, Cel + Cel two-stage hydrolysis was used to compare impacts of puffing at various temperatures (80 to 120°C) and pressures (0.5 to 1.0MPa) on FOY. In addition, to prevent emulsion involved by hydrolysate, salt iron was added to precipitate polysaccharide according to our previous publication (Meng et al., 2018; Peng et al., 2019). The selection for range of puffing parameter (temperature and pressure) referred to those reported by Yang et al. (2022) and Zhang et al. (2021) with minor modifications. The results were showed in Fig. 3., for dry-heating puffing, FOYs increased from 20.52–23.16% as puffing pressure increased from 0.5 MPa to 1.0 MPa. In view of higher free oil recovery of 93.0% (yielding 23.16%) by puffing at 1.0 MPa, no further increased pressure was considered, or which would result in much loss of active compound in gardenia seeds (refer to Table 3). When wet-heat puffing was performed with pressure was set at 1.0 MPa, the puffing temperature point is between 80 to 120°C, and corresponding FOYs fall into range of 20.38–21.76%. The optimal puffing pressure was 90°C, the corresponding FOY is 21.76%, which is slightly lower than that for dry-heating puffing. Both are higher than that of non-pre-treatment group with value of 68.44% and 58.25% higher, respectively. Besides of puffing, microwave (Gai et al., 2013; N. Liu et al., 2022), ultrasonic (N. Liu et al., 2022; Liu et al., 2019), extrusion (Jung et al., 2009), high pressure (Yuanchao Zhang et al., 2021) are also commonly used pretreatment method for oil preparation by AEE. It was reported that these methods enhanced oil extraction, with increases in oil yield varied from 8.3 to 44.4% (Ferreira et al., 2022). This highlighted the advantage of high performance for puffing pre-treatment compared with other methods with respect to oil preparation by AEE. Puffing technique was also mature and easy to scale up industrially. It is also suitable for samples with high moisture content, unlike other pre-treatment methods that require deeper drier material (Sun et al., 2021).

Table 3
Effects of different pre-treatments on the content of bioactive compounds in gardenia seeds

Peak	RT (min)	ESI-MS	Identification	Bioactive compounds content		
				Non-pre-treatment	DP pre-treatment (1.0MPa)	WP pre-treatment (90°C)
1	1.967	353 [M – H] [–]	Chlorogenic acid	0.47 ± 0.04 ^a	0.41 ± 0.02 ^b	0.23 ± 0.005 ^c
2	2.808	595 [M + HCOO] [–]	Genipin-1-gentiobioside	1.65 ± 0.07 ^a	1.54 ± 0.30 ^a	1.53 ± 0.008 ^a
3	3.343	433 [M + HCOO] [–]	Geniposide	7.68 ± 0.06 ^a	6.86 ± 0.37 ^b	6.83 ± 0.05 ^b
4	5.438	609 [M – H] [–]	Rutin	0.63 ± 0.13 ^a	0.57 ± 0.02 ^b	0.59 ± 0.002 ^{ab}
5	7.318	975 [M – H] [–]	Crocin	10.45 ± 0.07 ^a	6.67 ± 0.06 ^b	9.20 ± 0.02 ^c
Values were the mean ± SD of three gardenia seed oils which was analyzed in triplicate.						
The average values in the same row followed by the same superior letters were not significantly different (<i>p</i> > 0.05)						

3.4. Microstructure morphology of gardenia seeds puffed under various conditions

The morphology of microstructure for gardenia seeds puffed by various ways were characterized by using the field emission scanning electron microscope, and shown in Fig. 4. It was observed that electron micrographs for raw gardenia seed presents a compact structure and a smooth surface. After puffing, the microstructures for gardenia seeds became rough, full of irregular pore sizes and occasional large cavities, indicating that the rapid evaporation of water resulted from a sudden pressure drop by puffing produces a strong tearing force, which broke down the

microstructure of gardenia seeds and exposed large ratio surface area for enzymatic hydrolysis. Obviously, dry-heating puffing creates more destructive power in comparison with wet-heating puffing, and expansion degree increased as puffing pressure raised from 0.6MPa to 1.0MPa. For wet-heating puffing at 1.0MPa, less difference in microstructure for oilseeds was observed when temperature is increased between 90 and 100°C, however it seems that puffing at 90°C produce more rough structure which is more suitable for oil extraction by AEE (Wen et al., 2022).

3.5. Surface area, pore volume, and pore size of puffed gardenia seeds

The porous structure of seeds had a positive effect on the contact between enzymes and active sites during AEE process. Figure 5 showed the N₂ adsorption–desorption isotherm for gardenia seeds under DP pre-treatment, WP pre-treatment and non-pre-treatment. According to the IUPAC classification, all groups of gardenia seeds exhibited characteristic type-IV hysteresis loop, related to the mesoporous structures (Wang et al., 2022). Based on the BET analysis, the results of BET surface area, pore volume, and pore size were listed in Table 4. Compared with un-puffed seeds (0.3336m² g⁻¹), seeds under 1.0MPa DP pre-treatment (1.4219m² g⁻¹) exhibited a much larger BET surface area and similar rule was found for pore volume, indicating more porous property. This was due to the extreme explosion conditions which provided the seeds with DP pre-treatment (1.0MPa) with excellent porosity, thus the enhanced performance of DP pre-treatment was verified from effective destroys of the cell structure of gardenia seeds, Moreover, the average pore radius of DP pre-treatment seeds was smaller than that of non-pre-treatment seeds by 1.7%-7.5%, which might be explained by the dense structure of DP pre-treatment seeds at the surface (Huang et al., 2022).

Table 4
BET analysis of the gardenia seeds under different pre-treatment methods

Properties	Non-pre-treatment	DP pre-treatment		WP pre-treatment	
		0.6MPa	1.0MPa	90°C	100°C
BET surface area (m ² g ⁻¹)	0.3336	1.3063	1.4219	0.7139	0.7112
Pore volume (cm ³ g ⁻¹)	0.000344	0.001247	0.001443	0.00094	0.000791
Average pore diameter (nm)	4.1284	3.8197	4.0588	5.265	4.4477

3.6. HPLC-MS/MS analysis of the heat-sensitive bioactive compounds in puffed gardenia seeds

The preparation of gardenia yellow pigment is the primary application for gardenia fruit. While the functional components like crocin in gardenia seed are thermally unstable. High temperature and high pressure environment generated during puffing may be detrimental. Hence, its specific impact on active components should be clarified. Here, the main functional components in raw gardenia seeds and puffed gardenia seeds were extracted by 60% isopropanol, and the main products including chlorogenic acid (Rt 1.967min), genipin-1-gentiobioside (Rt 2.808min), geniposide (Rt 3.343min), rutin (Rt 5.438min), crocin (Rt 7.318min) were identified using HPLC-ESI-MS, and quantified with HPLC coupled diode array detector by monitoring absorption at 240nm and 440nm for geniposide and crocin. The chromatograph and composition were shown in Fig. S1. The impact of puffing ways on their composition was showed in Table 3. Except for Genipin-1-gentiobioside, all the active components including geniposide, crocin, rutin and chlorogenic acid decreased significantly through puffing pre-treatment against non-pre-treatment group. The worst loss was observed for chlorogenic acid, with values of 51.1% and 12.8% for wet-heating puffing and dry-heating puffing, indicating its sensitivity to damp heat than to dry heat. On the contrary, crocin is more prone to be destroyed upon dry heat puffing, with content decreased to 6.67% from 10.45% (control group), compared to 9.20% for wet heat puffing. Geniposide, similar with rutin, about 10% loss against control group was found through puffing pretreatment regardless of puffing ways.

In addition, the levels of geniposide and crocin in hydrolysis solution released by AEE process were determined and presented in Table 2. With geniposide and crocin in hydrolysate and those extracted into isopropanol considered, their sum of amount is equivalent to those in gardenia seeds, indicating that geniposide and crocin were completely released by AEE. The highest contents of geniposide and crocin in hydrolysate of 1.52 ± 0.04% and 0.61 ± 0.03% respectively, were obtained for Pec hydrolysis using raw oilseeds, the secondary is for Cel + Cel group with values of 1.08% and 0.41%. Compared to raw gardenia seeds, puffing resulted in lower levels of crocin in hydrolysate by AEE regardless of hydrolysis ways, and much low level of crocin in hydrolysate for dry-heating puffing than for wet-heating puffing (about 20% lower), which also supported that puffing resulted in observable loss of crocin. While the level of geniposide had not changed that much as crocin. These findings also verified the analytical results of HPLC to some degree.

3.7. Characterization of gardenia seed oil prepared by various methods

The yields of the gardenia seed oil by various preparation methods and their fatty composition, active components, and quality index. The color of all the oil extracted by various methods presents light yellow to yellow. For all the oil preparation method, Soxhlet extraction gave the highest yield of 25.45%, CPE produced the lowest yield of 10.44%. The oil yield for AEE is much higher than pressing method, with values of 13.75%, 21.76% and 23.16% upon raw seed, WP seeds and DP seeds. The PV for oils did not show significant difference among all the extraction methods, with values between 1.75–2.36 meq/g. For AV of oils, pressing method, solvent extraction and AEE with raw seed showed similar AVs of 1.25, 1.94 and 1.81 mg KOH/g, while puffed seed oils with higher AVs of 3.92 and 3.83 mg KOH/g. This is disadvantageous from view of oil quality (Xu et al., 2021). Slightly high AV should arise from hydrolysis of triglyceride under high-temperature and high-pressure conditions during puffing. In addition, content of active crocin and geniposide in oils are varied dependent on oil extraction methods. Higher levels of geniposide and crocin with value of 150.76 and 247.46 µg/g were obtained for oil prepared by AEE method, compared to levels of 14.13 and 102.16 µg/g for SE and 136.60 and 190.36 µg/g and for CPE method. Moreover, the composition of fatty acid in oil prepared by various methods were compared. Results showed that there is no significant difference among oils prepared by five extraction methods. Typically, gardenia seed oil mainly includes palmitic acid C16:0, oleic acid C18:1, linoleic acid C18:2, stearic acid C18:0 etc., with each content of 16.11–19.16%, 32.93–35.08%, 37.02–43.81% and 3.1–4.28%. Unsaturated fatty acids account for about 80% of total fatty acid, plus enrichment of active geniposide and crocin by oil, which indicated high nutritional value of gardenia seed oil.

4. Conclusions

To achieve high performance utilization of gardenia fruits, dehulling is necessary. For gardenia seeds, puffing pre-treatment facilitated the subsequent AEE for oil preparation and recovery of active crocin and geniposide. Two-stage enzymatic hydrolysis by cellulase was superior to any single enzymatic hydrolysis and other combined enzymatic hydrolysis. The highest oil yield of 23.16% were obtained when gardenia seeds puffed at 1.0MPa were hydrolyzed by Cel + Cel for 3h. This yield is far higher than that for pressing method, and close to solvent extraction method. Moreover, microstructure analysis by SEM and BET also agrees the point that puffing is an effective way to destroy the plant cell wall, leading to more opportunity to contact with enzymes during the hydrolysis process. There is no difference was observed for the gardenia seed oil prepared in this study with oil by other extraction method, in term of nutritional composition. In terms of safety, concerns about organic solvents residue in oil was omitted by this method. Hence, AEE coupled with puffing pre-treatment is a safe, highly efficient, environment friendly method for heath gardenia seed oil preparation, with a great industrialization prospect.

Declarations

Credit author statement

Chengyu Jin: Writing-original draft, Data curation, Validation and Experiment preparation; **Lingyun Wang:** Investigation and Software; **Xiaoying Liu:** Experiment preparation; **Yuanchao Lu:** Resource; **Ningxiang Yu:** Formal analysis; **Qin Ye:** Methodology; **Xiaohua Nie:** Language polishing; **Xianghe Meng:** Writing-review & editing, Conceptualization, Supervision, Funding acquisition.

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Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Figures

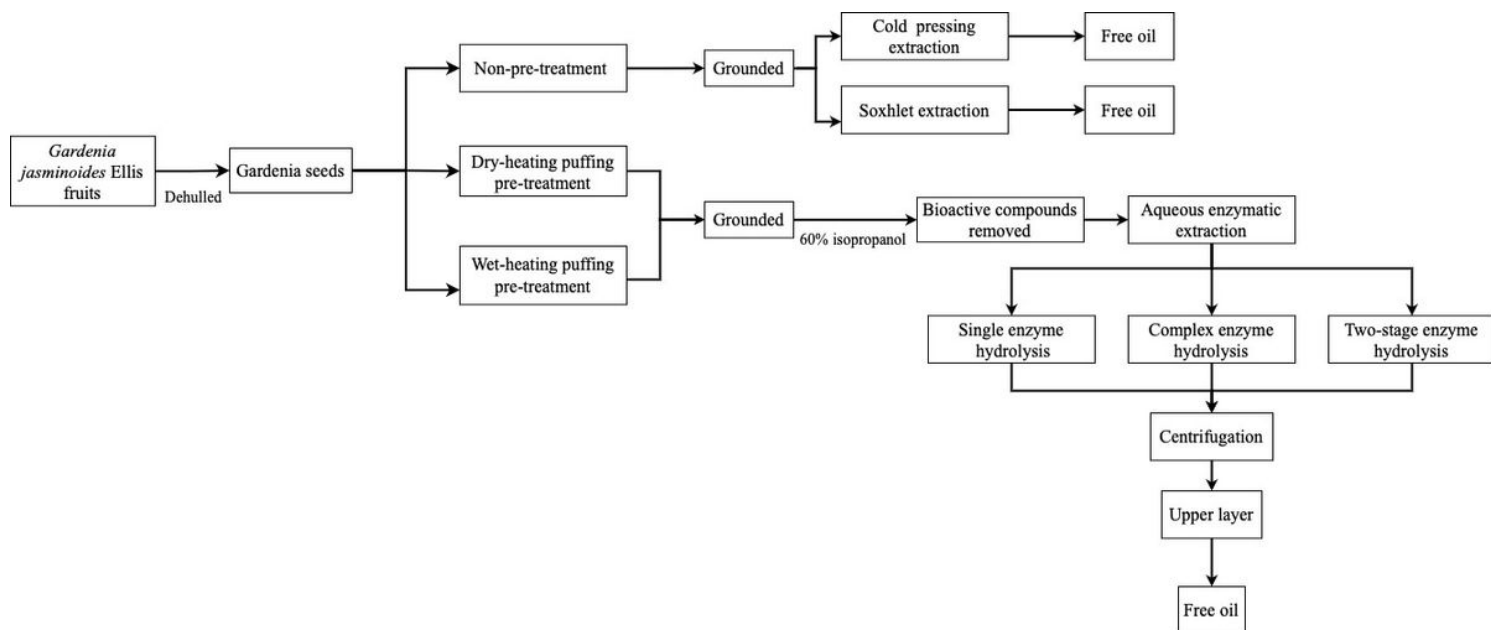


Figure 1

Process involved in the extraction of gardenia seed oil via CPE, SE and AEE methods.

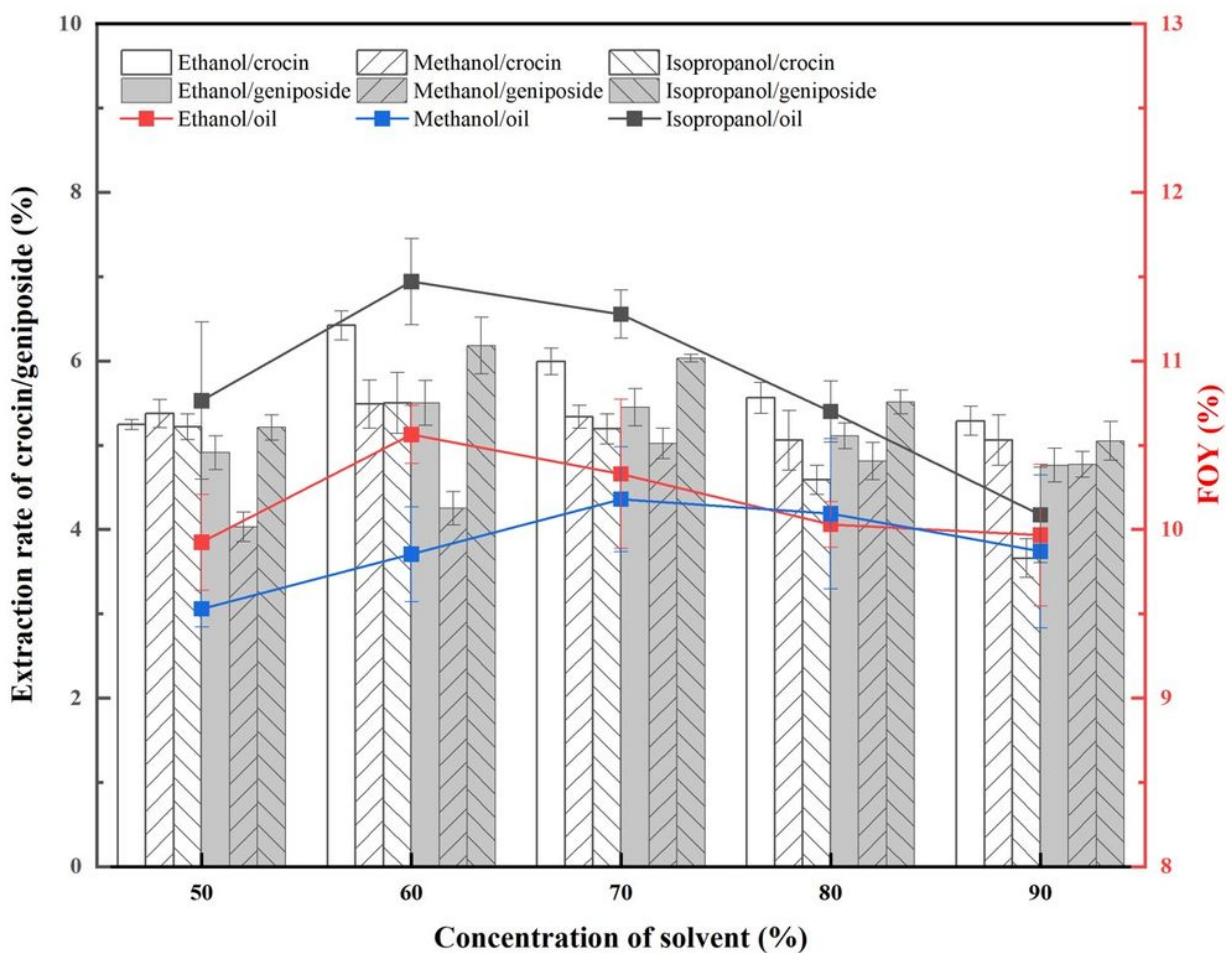


Figure 2

The yields for geniposide, crocin from gardenia seeds extracted by various solvent at different concentration and corresponding oil yields followed by aqueous cellulase hydrolysis

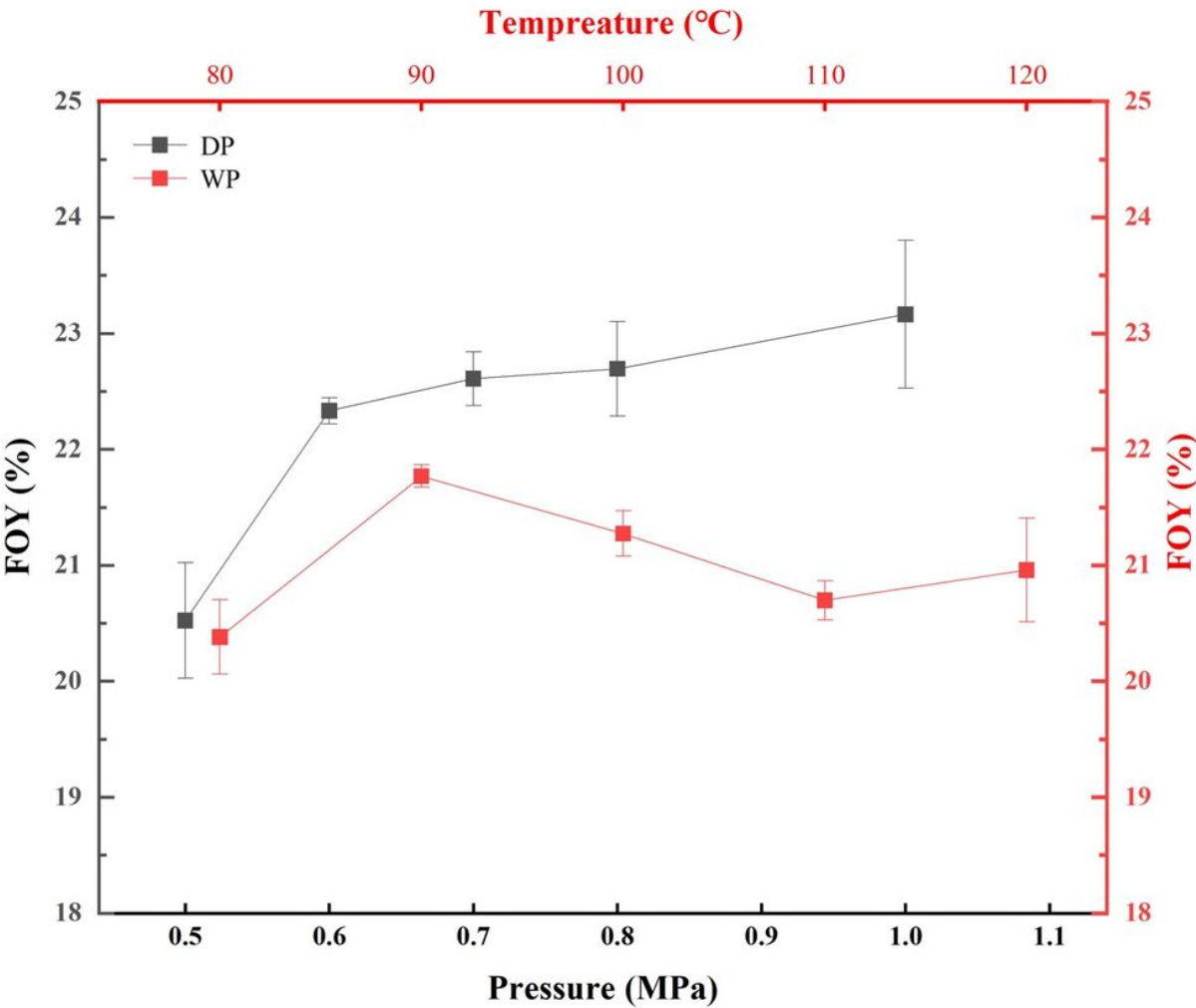


Figure 3

The yields of free oil from gardenia seed puffed at different conditions obtained by using AEE method

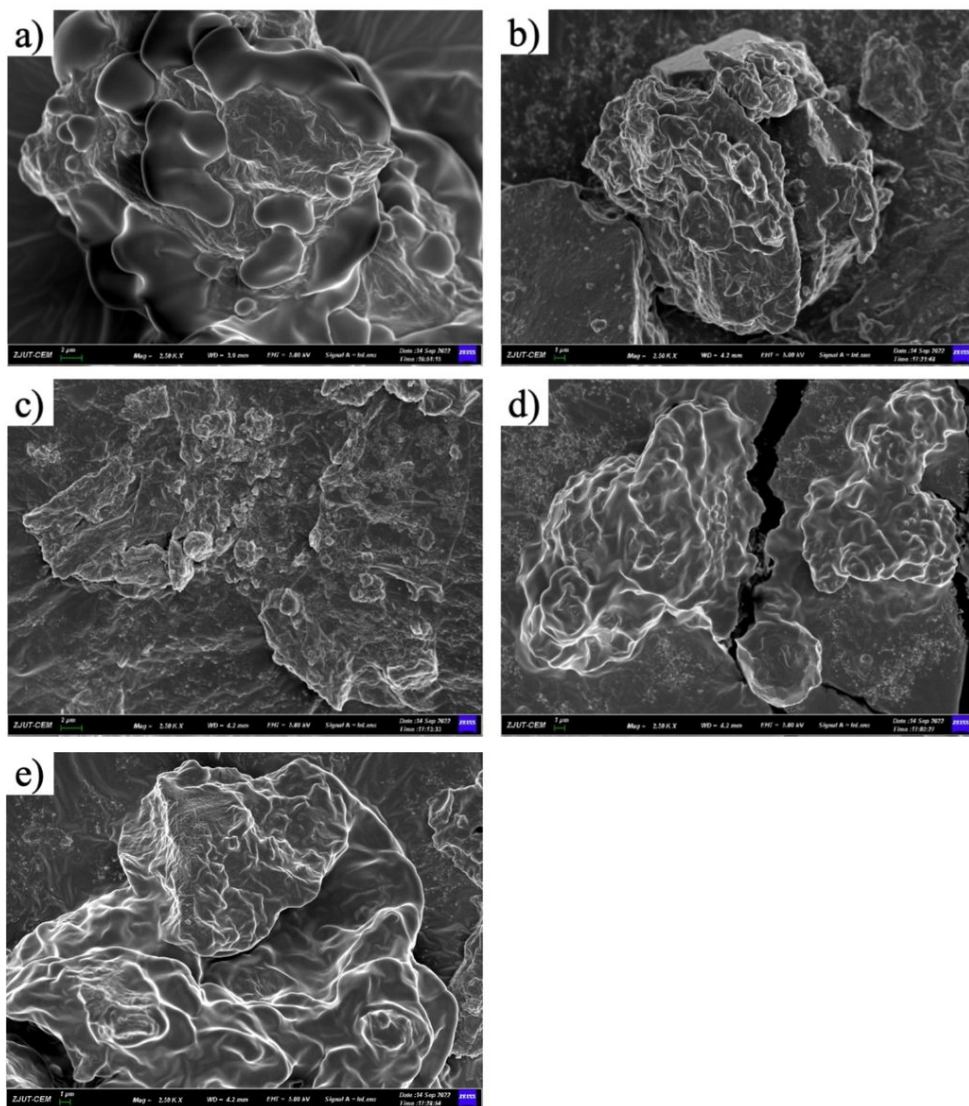


Figure 4

SEM images of gardenia seeds under different pre-treatment methods (a: non-pre-treatment, b: DP (0.6 MPa), c: DP (1.0 MPa), d: WP (90°C), e: WP (100°C))

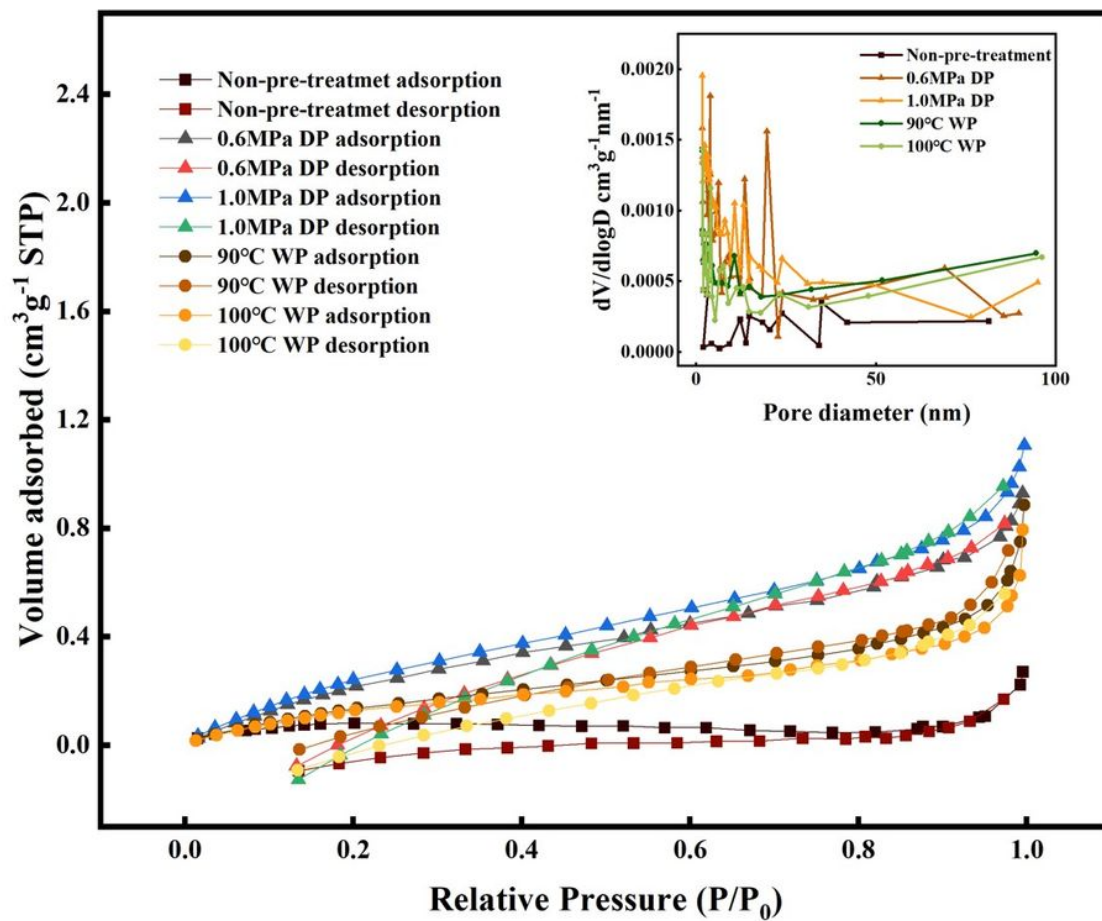


Figure 5

Nitrogen adsorption isotherms and pore size distribution of gardenia seeds under different pre-treatment methods.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial.docx](#)