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Evaluation of Antioxidant Activity of *Limonia acidissima* L. Leaf Using Ultrasound-Assisted Extraction

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Limonia acidissima L. (LA) is a fruit-bearing tree from the Rutaceae family that thrives in Tra Vinh province, Vietnam. It has been found that chemical compounds contained in LA leaves have antioxidant, antibacterial, anticancer, anti-inflammatory, and larvicidal properties. This study aimed to evaluate the antioxidant activity of leaf power ethanol-extracted using an ultrasound-assisted extraction technique. The input parameters of the process were optimized by the response surface methodology (RSM) including ethanol content (50 – 70 %), temperature (40 – 60 ºC), and time (30 – 50 min). The model response included total polyphenol content (TPC, Y_1), and radical scavenging capacity by DPPH (Y_2). The results show that the effect of the input parameters on the response of the extraction process was expressed by the quadratic equation with the high R^2 values of Y_1 (0.9827), and Y_2 (0.9644). The optimal conditions for the extraction process were achieved at X_1 = 59.7 %, X_2 = 49.4 ºC, and X_3 = 39.4 min. At this condition, the experimental value of TPC (85.79 ± 0.23 mg GAE/g dry weight, d.w) and DPPH (235.01 ± 0.79 µmol TEAC/g d.w) was not the significant difference compared to the predicted value. Furthermore, bioactive components from the leaf extract were quantified using modern GC-MS chromatography with the content of Stigmasterol (228.36 ± 4.95 ppm), Sitosterol (95.44 ± 3.3 ppm), Alpha-Pinene (60.40 ± 1.29 ppm). Antibacterial activity was tested against *Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa*, and *Staphylococcus aureus.*

1. Introduction

Limonia acidissima L., wood apple, is a plant with significant medicinal importance due to its various bioactive components. The leaves of LA power have composition like ash, fiber, fat, protein, carbohydrates (Nwauche et al., 2023), and many phytochemicals including alkaloids, saponins, cardiac glycosides, phenols, steroids, terpenoids, coumarins, sterols, and flavonoids (Wakchoure et al., 2023) which makes them suitable for both medicinal and food . Biological activity of chemical compounds isolated from leaves have anticancer, antimicrobial, antiulcer, antidiabetic, hepatoprotective, larvicidal properties (Murthy and Dalawai, 2019). Besides, LA leaves have been linked to antibacterial and antioxidant properties using various extraction solvents and methods.The traditional procedures (maceration and Soxhlet extraction) are commonly utilized in research labs. The current technologies include the extraction of microwave-assisted (MAE), ultrasound-assisted (UAE), and supercritical fluid (SFE), which are promising: The yield is boosted at a cheap cost (Azwanida NN, 2015). Besides, the choice of solvent also has a profound impact on the composition, yield and biological activity of the leaf extract. Ethanol is commonly used to extract a variety of polar and non-polar molecules that have high antibacterial properties while avoiding harmful residues (Mehmood et al., 2021). This study chose the ethanol and ultrasound-assisted extraction method to determine the phytochemical components, antioxidants, and anti-bacteria properties of LA leaf extract, which is the potential application orientation in food, medicine, and agriculture.

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2. Materials and methods

2.1 Chemicals

Foline-Ciocalteu, Na_2CO_3, C_2H_5OH were supplied by Merck, Germany; Acid gallic, Trolox, and DPPH were supplied by Sigma-Aldrich, USA. The other chemicals was purchased from Xilong Scientific Co., Ltd., Guangdong, China.

2.2 Preparation of Limonia acidissima L. leaves powder

LA leaves were collected from local farmers in CauNgang District, TraVinh Province, Vietnam. Leaves were washed with water to remove dust; then, drying the leaves in an oven. Crushing the dry leaves and sieve (500 mesh) to obtain powders of the same size and stored in plastic bags for research purposes.

2.3 Determination of UAE

The extraction of phenolic compounds from LA leaves was done in an ultrasonic generator bath (Powersonic 620, Hwashin, Korea). LA leaf powder was placed in a beaker containing ethanol solvent (50 – 70 %), sonication temperature (40 - 60 ºC), and sonication time (30 - 50 min), powder and solvent concentration ratio (1 : 30) were used in experimental design. The extract slurry was then centrifuged (9000 rpm) for 20 min. The supernatant was filtered through the whatman filter papers. The extract was kept at -20 ºC until analyzing total phenolic content (TPC), and radical scavenging activity by the DPPH method.

2.4 Determination of TPC

TPC was assessed using the Foline-Ciocalteu method (Darsini et al., 2013). Sample extracts were diluted with distilled water to fit on the standard curve range. Add the amount of sample (3 mL) and Foline Ciocalteu agent (0.5 mL) into the specimen tube. These tubes were kept in shadow. After 3 min of shaking, add 2 mL solution of 20 %, and incubate for exactly one minute in a bath of hot water (100 ºC). Tubes were then cooled and measured at 650 nm with a spectrophotometer (UV-1800, Shimadzu, Japan). The results were represented in milligrams (mg) of gallic acid equivalent (GAE) per gram of dry material.

2.5 Determination of DPPH

The radical scavenging assay was determined by the DPPH method (Brand-Williams et al., 1995) with a slightly modification. An amount of sample (0.3 mL) was mixed with 5.7 mL of a solution of DPPH in methanol (absorbance reached 1.1 ± 0.02 at 515 nm). The mixtures were forcefully vortexed. They were then kept in the dark (20 min). The absorbance value was detected at 515 nm using a spectrophotometer (UV-1800, Shimadzu, Japan), the decrease in DPPH of the sample was calculated as Eq (1)

$$\%inhibition = [1 - \frac{A_{sample}}{A_{control}}] * 100 \quad (1)$$

The standard curve showed an equation that was linear between 100 and 700 µmol/L Trolox and % inhibition, the concentration of Trolox equivalent was determined. The results were expressed in µmol Trolox equivalent antioxidant capacity per g of sample, d.w ((µmol TEAC/ g, d.w). The statistical analysis included three duplicates of each sample, with final findings provided as mean ± SD.

2.6 Identification of bioactive compounds by GC–MS analysis

Phytosterol and pinene were extracted from *Limonea acidissima* L. leaves. The extract was analyzed by gas chromatography with a flame ionization detector (GC-2030 Nexis, Shimadzu, Japan). The separation of phytosterol and pinene was performed using a DB-5MS column (Agilent Technology, USA) (30 m X 0.25 mm X 0.25 µm). The column oven temperature program was set to start at 50 ºC, hold for 2 min, then increase to 310 ºC at a rate of 40 ºC/min, and hold for another 10 min with a carrier gas flow of 1 mL/min. 1 µL volume was injected, using a 1 : 5 ratio setting at 300 ºC. Nitrogen was used as the carrier gas and makeup gas, while hydrogen was used as the fuel gas. The analytes were identified by retention time and confirmed by GC-MS according to the same parameters. The quantitation of the analytes was based on standard curves.

2.7 In vitro anti-microbial activity assay

The antibacterial efficacy of the leaf extract of LA was assessed using the diffusion well method (Phi et al., 2015). The extract was diluted with ethanol 40 %, which was used as a negative control. The bacteria were cultured on MHB medium and then diluted in a 0.85 % NaCl solution to achieve a turbidity similar to McFarland 0.5, which corresponds to a bacterial suspension of (1 - 3) x 10^8 CFU/mL. Utilize a sterile cotton swab to distribute the substance evenly on the MHA medium. Use a 9 mm diameter stainless steel tube to punch wells

on the MHA plate and load each well with 100 µL of extract. The plate is placed in an incubator set at a temperature of 37 ºC for a duration of 24 to 48 h, which varies based on the specific microbial strain being tested.

2.8 Experimental design

Response surface methodology (RSM) with central composite design (CCD) was used to optimize extraction parameters, including ethanol concentration (X_1: 50 – 70 %), sonication temperature (X_2: 40 - 60 ºC), and sonication time (X_3: 30 - 50 min).
Data analysis: The regression equation predicted the optimal process as shown in Eq(2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \beta_{ij} X_i X_j + e \quad (2)$$

In which, Y (the predicted response), β_0 (the constant coefficient), X_i X_j (the input variables); β_{ij} (the ijth interaction coefficient), β_{ii} (the ith quadratic coefficient), β_i (the ith linear coefficient), k (the number of factors), and e (the random error). The parameters were optimized by RSM using Design Expert software (version 11.0.7.1, Stat-Ease, Inc, Minneapolis, MN, USA).

3. Results and Discussion

3.1 Fitting the model

Response surface analysis fitted second-order polynomial model using experimental response variable values. The experimental and predicted values of the response did not differ significantly, suggesting a positive model. The model fitness was evaluated by the coefficient of determination (R^2), adjusted regression (R^2 adj) coefficients, and lack-of-fit value (Table 1).

Table 1: Correlation model by independent factors

Respones	2nd Order polynomial equation	Regresstion (*p*-value)	R^2	R^2 adj	Lack of fit
TPC	$-453.9339 + 6.4448X_1 + 13.2843X_2 + 0.8732X_3 - 0.0237X_1X_2 + 0.0267X_1X_3 - 0.028X_2X_3 - 0.0515X_1^2 - 0.1093X_2^2 - 0.015X_3^2$	0.0001	0.9827	0.9670	0.1194
DPPH	$-449.1337 + 16.7391X_1 + 3.0047X_2 + 5.8284X_3 - 0.0328X_1X_2 + 0.0356X_2X_3 - 0.1284X_1^2 - 0.0244X_2^2 - 0.0946X_3^2$	0.0001	0.9644	0.9386	0.8758

The R^2 of TPC (0.9827), and DPPH (0.9644) reached a high value, which demonstrated the model's fitness.

3.2 Influence of extraction parameters on total polyphenol content and antioxidant capacity

The ANOVA findings in Table 1 with a high *F-value* of TPC (0.1194), DPPH (0.8758), a low *p-value* of <0.0001 for both TPC and DPPH show the fitness of the model by the second-order quadratic according to temperature, ultrasonic exposure time, and solvent concentration.
The interaction X_1X_3 has positive effect, while X_1X_2 and X_2X_3 exhibits a negative effect on the total phenolic content. It was shown that TPC increased with an increase in concentration and extraction time. This relationship is depicted as parabolic on the response surface plot, similar to the model of the previous study (Biswas et al., 2023). The maximum value observed at TPC content (88.25 mg GAE/g d.w) when increasing ethanol concentration from 40 to 60 %, and time from 20 to 50 min. The above results are also similar to the previous study for the extract of *Vernonia amygdalina* leaves with 60 % ethanol resulting in the highest yield of TPC (110.34 mg GAE/g d.w) (Alara et al., 2019); higher than olive leaves (12.8 mg GAE/g) (Jaski et al., 2019). It suggests that different systems (ethanol-water) had various levels of efficiency in extraction, and adding an amount of water could increase extraction efficiency. This can be explained by the effect of water increasing the polarity of the solution of ethanol used during the extraction procedure, allowing for easier penetration into the plant matrix and resulting in greater polyphenol diffusion from the plant (Şahin and Şamli, 2013). On the contrary, TPC drops significantly in regions with increasing alcohol concentrations (> 60 %) and time (> 50 min). This could be explained that the pure solvent dehydrates plant tissue and desaturates phenolic compounds. These reasons result in lowering the overall phenolic concentration in the ultrasonic extract (Mojerlou and Elhamirad, 2018).

Figure 1 shows that the interaction X_2X_3 has a positive effect, while X_1X_2 exhibits a negative effect on the DPPH radical scavenging activity. The quadratic model of concentration and time was not significant with *p-values* > 0,05. The time and temperature interaction follow a similar vaulted shape on TPC. It was observed that with increasing temperature and extraction time, the yield of DPPH also increaseed from 195.21 to 238.22 µmol TEAC/g d.w. The optimal time and temperature values at 40 min and 50 ºC with the maximum DPPH (242.21 µmol TEAC/g d.w). When the temperature rises and the time longer, DPPH antioxidants tend to decrease gradually, caused by the impact of high temperature on biological components that are partially lost after birth, frailing the cell walls and significantly disintegrating or oxidizing the antioxidant activity. Similar results with Ashok Biswas' ultrasound exposure time of 37.20 min while the reflux extraction time of 120 min for Clinacanthus nutans Lindau leaves (Che Sulaiman et al., 2017) with the optimal DPPH radical scavenging activity (72.95 %) which is lower than this study (93.72 %). This demonstrates that ultrasonic extraction is more effective and takes a shorter time than traditional extraction. Temperature has an important role in controlling the procedure of extraction since it softens tissues, increases solubility and diffusion of compounds, reduces solvent surface tension, and so promotes wetting the biological substances (Jovanović et al., 2017) while vapor pressure increases, allowing the development of bubble cavitation at fewer acoustic extent and positively effecting the phenolic extraction efficiency. Using high temperatures for polyphenol extraction might cause solvent loss and oxidation, reducing thermally sensitive phenol molecules by hydrolysis or internal redox reactions (Sood and Gupta, 2015). Extraction yield increased with higher temperature and lower ethanol concentration. The extract's antioxidant effects may be increment due to the combination or synergistic activity of individual polyphenols, which are impacted by their chemical structure. As a result, the variance in polyphenol amount corresponded to the observed variation in antioxidant activity, indicating the positive correlation between polyphenolic and antioxidant content (Yumita et al., 2023). Antioxidant capacity varied significantly according to harvesting stage, genotype, species, and individual bushes (Venskutonis et al., 2016).

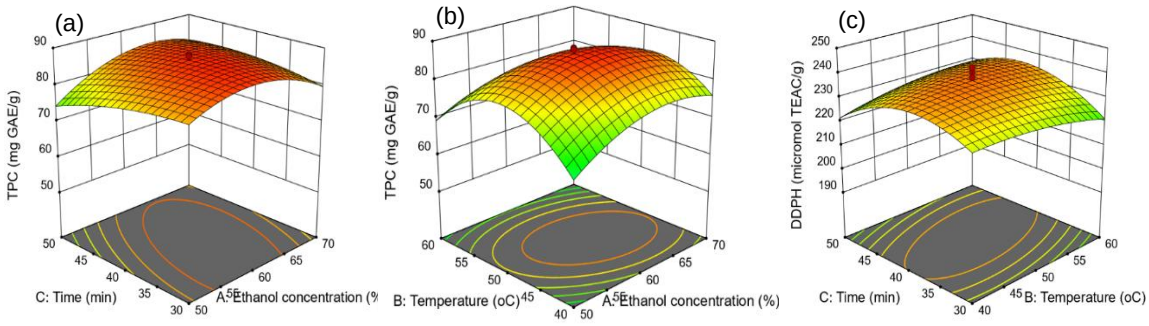

Figure 1: Response surface plot of the interactive effect of parameters of temperature, ethanol concentration and time on TPC (a, b) and DPPH (c)

3.3 Optimization responses and model verification

The model suggested that the optimal predicted value for the extraction process at the condition of 59.7 % ethanol at 49.4 ºC for 39.4 min. The highest predicted response for TPC of 86.198 mg GAE/g d.w and DPPH of 236.893 µmol TEAC/g d.w. was not a significant difference compared to the experimental value (TPC of 85.79 mg GAE/g d.w, and DPPH of 235.01 µmol TEAC/g d.w with inhibition 90.94 %). That proved the fitting of the predicted model. These results demonstrate that the extract from LA leaves contains more phenolic compounds and antioxidant activity than the extract from LA fruits as reported of TPC (0.22436 mg GAE/g), DPPH (inhibition 93.49 %) at the extraction condition of 52.68 % methanol, 47.89 ºC temperature, 39.8 min by RSM (Darsini et al., 2013). The results of this study also demonstrate that LA leaves contain higher TPC and DPPH than TPC (63.84 mg GAE/g d.w.) and DPPH (inhibition 90 %) in LA shells (Murakonda and Dwivedi, 2022).

3.4 GC-MS quantification of bioactive compounds in the optimized extract of Limonia acidissima L. leaves

The optimized extract's bioactive components were quantified using GC-MS chromatography, including alpha-pinene, stigmasterol, and sitosterol. Table 2 displays the retention time, area, and concentration of these substances, which were also found in the essential oils of LA leaves and fruits (Murthy and Dalawai, 2019). Phytosterol compounds accounted for higher content than terpene compounds, as in research (Mukhtar et al., 2018). The chemical stigmasterol has the highest amount (228.36 ± 4.95 ppm). This result is higher than the previous study's 32.2 ppm in sour eggplant leaf extract (Fadhli et al., 2023), which has antimicrobial, antitumor, and antioxidant activities (Zhang et al., 2022).

Table 2: Quantification of bioactive compounds of leave extract by GC–MS analysis

Compound name	Retention time (min)	Area (mAU*min)	Concentration (ppm)
Alpha-Pinene	7.161	1476	60.40 ± 1.29
Stigmasterol	26.357	19682	228.36 ± 4.95
Sitosterol	27.172	2164	95.44 ± 3.3

3.5 In vitro anti-microbial activity assay

As demonstrated in Figure 2 and Table 3, the antibacterial activity of LA leaf ethanol extract was tested by determining the inhibition zone (mm) for *E. coli, S. enterica, P. aeruginosa, and S. aureus*. All Gram-negative bacteria are mostly resistant in extracts with concentrations of 350-700 mg/L (5 - 9 mm for *E. coli*, 6 - 11 mm for *S. enterica*, and 4 - 6 mm for *P. aeruginosa*). All the same, at a concentration of 350 mg/L, the extract was ineffective for *S. aureus* bacteria. Gram-positive bacteria have a lower inhibitory zone (3 - 4 mm) than Gram-negative microbe. The 40 % alcohol solvent concentration employed as a negative control showed no effect on any bacterium. The active plants or bioactive substances identified in this study were resistant to Gram-positive and Gram-negative bacteria (Hou et al., 2020).

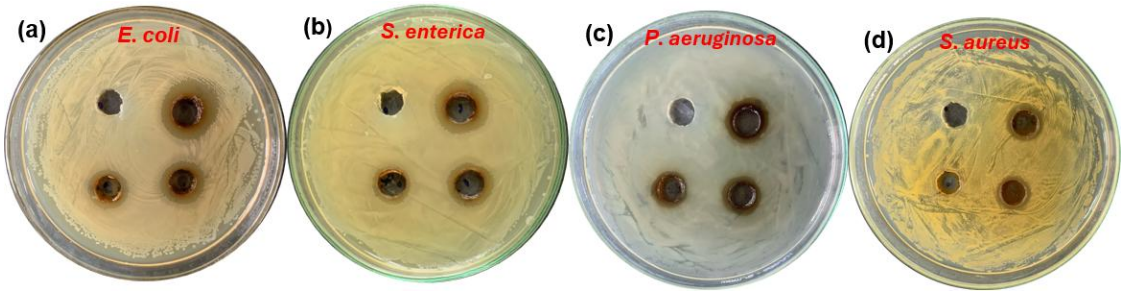

Figure 2: Image of antibacterial ring at various doses of LA leaf extract for gram-negative (a, b, c) and gram-positive (d) bacteria

Table 3: Diameter of the inhibition zone of the various extract concentrations against bacteria (mm)

	E. coli	*S. enterica*	*P. aeruginosa*	*S. aureus*
(1) 350 mg/mL	5	6	4	-
(2) 500 mg/mL	6	9	4	3
(3) 750 mg/mL	9	11	6	4
(-) Ethanol 40 %	-	-	-	-

4. Conclusions

RSM with the quadratic polynomial model is suitable to optimize the parameters of the extraction process in TPC and DPPH of LA leaves. The chromatography proves the presence of a strong sterol and terpene compounds in the leaf extract received from the extract process at the optimal condition of the predicted model. The extract of the leaves also demonstrated inhibition against all Gram-positive and Gram-negative microorganisms, which have promising applications in industries such as agriculture, pharmaceutical, medicine and food.

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