Songklanakarin J. Sci. Technol.
44 (1), 40-44, Jan. - Feb. 2022

http://www.sjst.psu.ac.th

Original Article

Chemical composition, antioxidant and antibacterial activities of ultrasound-assisted extract of *Annona squamosa* L. leaves♣

Pornpun Siramon[1*], and Thitima Wongsheree[2]

[1] *King Mongkut's University of Technology Thonburi* (*Ratchaburi Campus*), *Rang Bua, Chom Bueng, Ratchaburi, 70150 Thailand*

[2] *Institute for Scientific and Technological Research and Services, King Mongkut's University of Technology Thonburi, Thung Khru, Bangkok, 10140 Thailand*

Received: 18 March 2021; Revised: 29 June 2021; Accepted: 2 July 2021

Abstract

The bioactive compounds were extracted from *Annona squamosa* leaves using ultrasound-assisted extraction (UAE) and the optimal extraction conditions were determined. It was found that the extraction of the sample with 50% (aq) of ethanol at 50 ºC for 60 minutes provided the highest crude extract yield of 30.50% on a dry basis. Further, this condition gave a total phenolic content of 307.67 µg GAE/g DW and total flavonoid content of 16,893.92 µg CE/g DW. The crude extract obtained from this condition gave the best results for antioxidant activities with the IC_{50} values of 947.99 and 620.89 µg/mL by DPPH and ABTS assays, respectively. The chemical composition of the crude extract was analysed by LC-ESI-MS, Rutin and Norisocorydine were detected. Finally, the antibacterial properties of the crude extract were tested against three human pathogenic bacterial strains. Results revealed that the crude extract exhibited antibacterial activities against all tested strains.

Keywords: *Annona squamosa* L., antibacterial activity, antioxidant activity, chemical composition, ultrasound-assisted extraction (UAE)

1. Introduction

Annona squamosa Linn. belongs to the Annonaceae family, commonly known as custard apple. This plant is a semi-evergreen tree native to the West Indies and can be cultivated throughout many Asian countries, including Thailand. Different parts of *A. squamosa* were traditionally used in folkloric medicine for the treatment of various illnesses. *A. squamosa* extracts were reported to have anticancer, antioxidant, anti-inflammatory, and antimicrobial properties (Salman, Ramasamy, & Mahmood, 2018). The various chemical constituents isolated from leaves, stems, and roots of the plant included glycoside, alkaloids, saponins, flavonoids, tannins, carbohydrates, proteins, phenolic compounds, phytosterols, and amino acids (Pandey & Barve, 2011).

Nowadays, the use of ultrasound-assisted extraction (UAE) for separating bioactive compounds from plants or various agricultural by-products has increased substantially. Its advantages over conventional extraction methods include significantly reduced extraction time and low temperature used, which help to prevent thermal damage to heat-sensitive products during the extraction process (Rodrigues, Pinto, & Fernandes, 2008).

The aim of this research was to determine the optimal extraction conditions for the bioactive compounds from an abundant agricultural residue *A. squamosa* leaves by ultrasound-assisted solvent extraction. The properties of crude extracts from each extraction condition, namely the total phenolic and total flavonoid contents, and the antioxidant activities were investigated. The chemical compositions of the

♣Peer-reviewed paper selected from The 1st International Conference on Sustainable Agriculture and Aquaculture (ICSAA-2021)
*Corresponding author
Email address: siramon_p@hotmail.com

crude extract from the optimal extraction condition were analyzed using a liquid chromatography-electrospray ionisation-mass spectrometer (LC-ESI-MS). Antibacterial activities against three human pathogenic bacterial strains were also investigated.

2. Materials and Methods

2.1 Raw material

A. squamosa L. leaves were collected from a plantation in Chom Bueng District, Ratchaburi Province, Thailand. The leaf sample was cleaned, cut into smaller pieces, and then oven-dried at 40 ºC until the moisture content of the sample was less than 10%. The dried sample was further ground and stored in an airtight container for further analysis.

2.2 Optimal extraction conditions for bioactive compounds from the *A. squamosa* leaf powder by UAE

2.2.1. Influence of solvents upon extraction

The extraction of bioactive compounds from *A. squamosa* leaf powder was conducted by Maceration. The samples were subjected to extract with three different concentrations of ethanol [50%, 70%, and 95% (aq) of ethanol] using the ratio of solvent-to-sample of 100 (v/w). The sample was soaked in a different solvent at 30 °C for three days before filtering the resulting mixture using filter paper, whereupon the filtrate was evaporated until dry. The yield could then be measured. Further examination was carried out with the crude extracts once dried in order to measure the total phenolic contents according to the method of Singleton, Orthofer, & Lamuela-Raventos (1999). This helped in identifying the most appropriate solvent to be used to extract the bioactive compounds.

2.2.2 Conditions for extraction by UAE

The optimal operating conditions (time and temperature for extraction) for the extraction of the bioactive compounds from *A. squamosa* leaf powder were conducted using an ultrasonic cleaning bath (Bandelin sonorex digitec, DT 510 H, 35 kHz, 16 W). Samples were extracted with the optimal solvent (results obtained from 2.2.1) using the ratio for solvent-to-sample of 100 (v/w). An ultrasonic bath was used for UAE across a range of different temperatures (30 and 50 °C) and durations of time (30, 60, and 120 minutes). The extraction yield and total phenolic contents were determined using the same procedures described in 2.2.1. The flavonoid contents in the crude extracts were determined according to the method of Wolfe, Wu, & Liu (2003). The antioxidant activity of crude extract from each extraction condition was further investigated through comparison of two different radical scavenging assays. The first is the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, which was determined according to the method recommended by Siramon & Ohtani (2007). The second is the 2,2'-azino-bis (3-ethylbenzothia zoline-6-sulphonic acid) (ABTS) method, which is determined according to Re *et al*. (1999). The measured scavenging ability for both assays was stated in the form of IC_{50} values, representing the concentration necessary to halt 50% of each assay activity. The control standards in these assays were butylated hydroxytoluene (BHT) and α-tocopherol.

2.3 Chemical composition of crude extract

The chemical composition of *A. squamosa* crude extract from the optimal extraction conditions was analyzed by Liquid Chromatography-Electrospray Ionisation-Mass Spectrometer (LC-ESI-MS) (Agilent Technologies 6420 Triple Quad) in a positive ionisation mode. A ZORBAX Eclipse Plus C18 analytical column (4.6×100 mm, 3.5µm; Agilent) was used for LC separation. The solvent gradient HPLC analysis was applied using the modified method of Lee *et al*. (2008). The mobile phase consisted of solvents A and B. Solvent A was 0.1% glacial acetic acid in distilled water, and solvent B was 0.1% glacial acetic acid in ACN. The solvent flow rate was 1 mL min^{-1}, and the wavelength of the PDA was 280 nm. The injection volume was 20 µL of the sample, the linear gradient of HPLC solvent was as follows**:** B was increased from 8 to 10% for 2 min, then from 10 to 30% for 25 min, from 30 to 90% for 23 min, from 90 to 100% for 10 min, and kept at 100% for 5 min, before being returned to the initiation state. The authentic standard Rutin was used to confirm the fragmentation pattern in the sample. The full mass spectra were recorded in the 100-1500 *m/z* range.

2.4 Antibacterial activity evaluation

2.4.1 Preparation of extract solution

A stock solution of 204.80 mg/mL crude extract in dimethyl sulfoxide (DMSO) was prepared. The extract solutions were sterilized by passing them through a 0.45 µm membrane filter.

2.4.2 Microbial strains

The three human pathogenic bacterial strains used in this study were *Staphylococcus aureus* DMST 8840, *Staphylococcus epidermidis* DMST 15505, and Methicillin-resistant *Staphylococcus aureus* (MRSA) DMST 20651. The bacterial strains were grown and maintained on nutrient agar slant at 37 ºC for 24 hours. The inoculum size of each test strain was 10^8 bacteria/mL.

2.4.3 MIC and MBC

The minimum inhibitory concentration (MIC) of the extracts was determined according to the method of Rahman, Kuhn, Rahman, Olsson-Liljequist, & Mollby (2004) using the two-fold serial microdilution method. The tested extracts were added to a sterile Mueller Hinton broth and put onto microtiter plates before the diluted bacterial suspension was added. Each extract was assayed in triplicate. The bacterial suspensions were used as the positive control and extracts in broth were used as the negative control. The minimum bactericidal concentration (MBC) was determined according to Basri & Fan (2005) by a subculture of the well showing no apparent growth in a sterile agar plate. The lowest concentration showing no visible growth on agar subculture was taken as

MBC value. Antibiotic erythromycin was used as the standard.

2.5 Data analysis

The form of means ± standard deviations (SD) from studies were replicated in triplicate, while the analyses were performed using statistical software. The differences between means were assessed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT), with significance determined using p-values < 0.05.

3. Results and Discussion

3.1. Effect of solvent on bioactive compound extraction

The effects of solvent concentrations of 50%, 70%, and 95% (aq) ethanol are shown in Table 1. The concentration of solvent significantly affected the phenolic contents of the crude extracts. Ethanol (50%) aqueous solution was demonstrated to be the most effective solvent as it provided the highest percentage yield of extract (28.85% w/w on dry basis) and the highest total phenolic content (78.44 µg GAE/g dry weight of the sample).

3.2. Optimal conditions for extraction by UAE

The extraction temperature and time significantly affected the total phenolic contents, total flavonoid contents, and antioxidant activities of the samples, as shown in Table 2. Samples extracted from *A. squamosa* leaf powder with 50% (aq) ethanol (the optimal solvent) at 50 °C for 60 minutes in an ultrasonic bath gave the best results for percentage yield and antioxidant activities. These extraction conditions produced the highest percentage yield of the extract at 30.50 % w/w on DW and gave the total phenolic content of 307.67 µg GAE/g DW and total flavonoid content of 16,893.92 µg CE/g DW. The crude extract obtained from this condition gave the best results of antioxidant activities with the IC_{50} values of 947.99 and 620.89 µg/mL by DPPH and ABTS assays, respectively. The IC_{50} values for the synthetic antioxidant BHT and the natural antioxidant α-tocopherol were also reported for comparative purposes. The results showed that crude extract exhibited lower antioxidant activities than the standard controls by both methods. All the above results demonstrated that UAE, a potential alternative to conventional extraction methods, was able to obtain good quality extracts with high yields in short time and low temperature.

Table 1. Effect of solvents on the extraction yield and total phenolic content of *A. squamosa* leaf extract by maceration

Solvent	Yield (%)[1,2]	Total phenolic content (µg GAE /g)[1,2]
50% (v/v) ethanol	28.85 ± 0.87[a]	78.44 ± 2.29[a]
70% (v/v) ethanol	26.81 ± 0.53[b]	73.33 ± 1.81[b]
95% (v/v) ethanol	22.22 ± 0.01[c]	74.31 ± 0.94[b]

[1]Values are means of three replications ± SD. Numbers followed by different alphabetical among each column are significantly different (P < 0.05). [2]Data were based on a dry weight basis.

3.3. Identification of chemical composition

The total ion chromatogram of the *A. squamosa* crude leaf extract is shown in Figure 1. The mass spectrum analysis (Figure 2) revealed that Peak 1 was identified as Quercetin-3-O-rhamnosylglucoside (Rutin) (MW 610) due to the presence of the base peak at *m/z* 302.8, which was detected and identified as Quercetin (Pawar & Nasreen, 2018). Peak 2 showed $[M-H]^+$ ion of *m/z* 327.9, which was identified as Norisocorydine (mw 327) (Pandey & Barve, 2011).

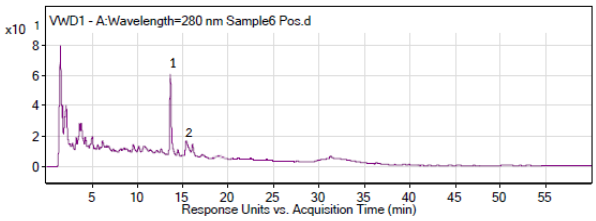

Figure 1. Total ion chromatogram of the *A. squamosa* leaf extract

Table 2. Extraction yield, total phenolic and total flavonoid contents of *A. squamosa* leaf extract using 50% (v/v) ethanol as the extraction solvent at different temperatures and duration by UAE.

Extraction conditions		Yield (%)[1,2]	Total phenolic content (µg GAE /g)[1,2]	Total flavonoid content (µg CE /g)[1,2]	IC_{50} (µg/mL)[3]	
Temp. (^{0}C)	Time (min)				DPPH	ABTS
30	30	28.03 ± 0.37[c]	315.30 ± 1.47[ab]	17,795.31 ± 1.24[b]	1,172.31±1.31[e]	713.12±1.54[c]
	60	28.59 ± 0.39[c]	312.68 ± 1.31[c]	15,697.88 ± 1.44[e]	1,022.87±1.21[c]	696.28±1.17[b]
	120	29.49 ± 0.29[b]	302.43 ± 1.57[e]	15,557.63 ± 1.38[f]	977.08±1.03[b]	822.29±1.29[e]
50	30	29.62 ± 0.22[b]	314.82 ± 1.15[bc]	18,260.18 ± 1.18[a]	1,026.68±1.57[c]	826.60±1.13[f]
	60	30.50 ± 0.35[a]	307.67 ± 1.27[d]	16,893.92 ± 1.37[d]	947.99±1.44[a]	620.89±1.56[a]
	120	30.79 ± 0.29[a]	317.45 ± 1.23[a]	17,351.02 ± 1.56[c]	1,072.13±1.42[d]	780.30±1.30[d]
Butylated hydroxytoluene (BHT)					180.32	215.45
α-Tocopherol					383.14	375.01

[1]Values are means of three replications ± SD. Numbers followed by different alphabetical among each column are significantly different (P < 0.05). [2] Data were based on a dry weight basis. [3]Data were based on crude sample weight.

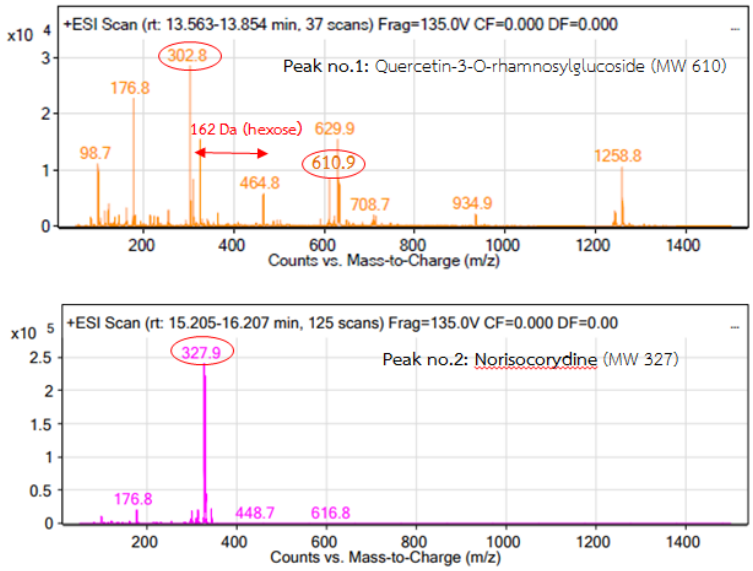

Figure 2. Mass spectra of the identified peaks in the *A. squamosa* leaf extract

3.4. Determination of antimicrobial activities

The antibacterial activities of the crude extract were tested against 3 human pathogenic strains**:** *Staphylococcus aureus* DMST 8840, *Staphylococcus epidermidis* DMST 15505, and Methicillin-resistant *Staphylococcus aureus* (MRSA) DMST 20651. These test strains involved gram-positive bacteria, which cause dermal infection. Methicillin-resistant *S. aureus* (MRSA) is a *S. aureus* that is resistant to methicillin and beta lactam (Enright *et al*., 2002). It has also been reported to resist other kinds of antibiotics as well, such as erythromycin, genatmacin, and tetracyclines (Khan, Faisal, & Hasnain, 2010). The tested results in Table 3 showed that *A. squamosa* crude leaf extract exhibited antibacterial activity against all tested bacterial strains with minimal inhibitory concentration (MIC) values in the range of 12.80-25.60 mg/mL and minimal bactericidal concentration (MBC) value of 25.60 mg/mL.

These biological properties were related to the presence of a flavonol glycoside**:** Rutin and the aporphine alkaloids**:** Norisocorydine. Recently, a glycoside form of Quercetin- Rutin is extensively studied for antimicrobial activity against various strains of bacteria (Adamczak, Ożarowsk, & Karpiński, 2019; Amin *et al*., 2015; Gullón *et al*., 2017). It has been reported that the antibacterial action of Rutin was mainly due to the inhibition of DNA isomerase IV (Ganeshpurkar & Saluja, 2017), and two hydroxyl substituents on C-5 and C-7 of ring A on its structure exerted rapid bactericidal action, resulting in bacterial membrane damage, leakage of intracellular compounds, and protein coagulation (Xie *et al*., 2017). Several research studies have been reported that the major alkaloid constituents isolated from *A. squamosa* leaves were identified as Corydine, Norisocorydine, Norlaureline, Norcodeine, Oxanalobine and Aporphine (Bhakuni, Tewari, & Dhar, 1972; Pandey & Barve, 2011). These alkaloid fractions also showed significant antibacterial effects against the test bacterial strains. Furthermore, the effects on bacterial cells treated with alkaloid extracts of *A. squamosa* leaves were examined using scanning electron microscope. It was found that the mode of action of alkaloidal on bacterial cells showed changes in cell morphology such as swelling of cells, rupture in cell wall, cell lysis and apoptosis (Shami, 2017).

Results of these studies demonstrated that *A. squamosa* leaf extract was found to exert multiple antibacterial functions and exhibit antibacterial activities against all test bacterial strains including MRSA. Therefore, *A. squamosa* leaf extract, plant-derived antimicrobials, could be used for developing effective, natural, and safe antimicrobials in future.

Table 3. Minimal inhibitory concentration (MIC, mg/mL) and minimal bactericidal concentration (MBC, mg/mL) for the crude extract and standard Erythromycin

Bacterial strains	*S. aureus*		*S. aureus* (MRSA)		*S. epidermidis*	
Concentration	MIC	MBC	MIC	MBC	MIC	MBC
Crude extract	12.80	25.60	12.80	25.60	25.60	25.60
Erythromycin	0.04	0.04	0	0	0.02	0.02

Note 0 = no inhibition

4. Conclusions

The optimal extraction conditions of *A. squamosa* leaves by UAE were found to be ethanol concentration of 50%, extraction temperature of 50 ºC, and extraction time of 60 minutes. These conditions provided the highest crude extract yield of 30.50% on a dry basis and gave the total phenolic and total flavonoid contents of 307.67 µg GAE/g DW and 16,893.92 µg CE/g DW, respectively. Rutin and Norisocorydine were identified as the major compositions in the crude extract. The antioxidant assay results showed that the crude extract exhibited antioxidant activity with the IC_{50} values of 947.99 µg/mL by DPPH and 620.89 µg/mL by ABTS, respectively. The crude extract also exhibited antibacterial activity against all tested bacterial strains with

minimal inhibitory concentration (MIC) values in the range of 12.80-25.60 mg/mL and minimal bactericidal concentration (MBC) value of 25.60 mg/mL. From the test results, it could be concluded that *A. squamosa* leaves, an abundant agricultural residue rich in phenolics and flavonoids, exhibited effective antioxidant and antibacterial activities, which could be used as an alternative natural source of antioxidants and antibacterial agents.

Acknowledgements

This research was supported by The RSPG-KMUTT, Thailand.

References

Adamczak, A., Ożarowsk, M., & Karpiński, T. M. (2019). Antibacterial activity of some flavonoids and organic acids widely distributed in plants. *Journal of Clinical Medicine, 9*(1), 109-125.

Amin, M. U., Khurram, M., Khattak, B., & Khan, J. (2015). Antibiotic additive and synergistic action of rutin, morin and quercetin against methicillin resistant *Staphylococcus aureus*. *BMC Comple mentary and Alternative Medicine, 15*(1), 1-12.

Basri, D. F., & Fan, S. H. (2005). The potential of aqueous and acetone extracts of galls of *Quercus infectoria* as antibacterial agents. *Indian Journal of Pharmacol*ogy, *37*(1), 26-29.

Bhakuni, D. S., Tewari, S., & Dhar, M. M. (1972). Aporphine alkaloids of *Annona squamosa*. *Phytochemistry, 11*, 1819-1822.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences, 99*(11), 7687-7692.

Ganeshpurkar, A., & Saluja, A. K. (2017). The pharma cological potential of rutin. *Saudi Pharmaceutical Journal, 25*(2), 149-164.

Gullón, B., Lú-Chau, T. A., Moreira, M. T., Lema, J. M., & Eibes, G. (2017). Rutin**:** A review on extraction, identification and purification methods, biological activities and approaches to enhance its bio availability. *Trends in Food Science and Technology, 67*, 220-235.

Khan, A., Faisal, S., & Hasnain, S. (2010). The continuing threat of methicillin-resistant *Staphylococcus aureus*-past present future. *Journal of Scientific Research, 40*, 37-45.

Lee, S. J., Kim, J. J., Moon, H. I., Ahn, J. K., Chun, S. C., Jung, W. S., . . . Chung, I. M. (2008). Analysis of isoflavones and phenolic compounds in Korean soybean [*Glycine max* (L.) Merrill] seeds of different seed weights. *Journal of Agricultural and Food Chemistry, 56*(8), 2751-2758.

Pandey, N., & Barve, D. (2011). Phytochemical and pharmacological review on *Annona squamosa*l Linn. *International Journal of Research in Pharmaceutical and Biomedical Science, 2*(4), 1404-1412.

Pawar, D. S., & Nasreen, S. (2018). HR-LCMS of phytoconstituents and antifungal activity of medicinal plant. *Journal of Medicinal Plants Studies, 6*(1), 173-176.

Rahman, M., Kuhn, I., Rahman, M., Olsson-Liljequist, B., & Mollby, R. (2004). Evaluation of a scanner-assisted colorimetric MIC method for susceptibility testing of gram-negative fermentative bacteria. *Applied and Environmental Microbiology, 70*(4), 2398-2403.

Re, R., Pellegrini, N., Proteggente, A, Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231-1237.

Rodrigues, S., Pinto, G. A. S., & Fernandes, F. A. N. (2008). Optimization of ultrasound extraction of phenolic compounds from coconut (*Cocos nucifera*) shell powder by response surface methodology. *Ultrasonics Sonochemistry, 15*, 95-100.

Salman, H. A., Ramasamy, S., & Mahmood, B. S. (2018). Detection of caffeic and chlorogenic acids from methanolic extract of *Annona squamos*a bark by LC-ESI-MS/MS. *Journal of Intercultural Ethno pharmacology, 7*(1), 76-81.

Shami, A. M. (2017). The effect of alkaloidal fraction from *Annona squamosa* L. against pathogenic bacteria with antioxidant activities. *Pharmaceutical Sciences, 23*, 301-307.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299*, 152-178.

Siramon, P., & Ohtani, Y. (2007). Antioxidative and antiradical activities of *Eucalyptus camaldulensis* leaf oils from Thailand. *Journal of Wood Science, 53*(6), 498-504.

Wolfe, K., Wu, X., & Liu, R. H. (2003). Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry, 51*, 609-614.

Xie, Y., Chen, J., Xiao, A., & Liu, L. (2017). Antibacterial activity of polyphenols: Structure-activity relationship and influence of hyperglycemic condition. *Molecules, 22*, 1913-1924.