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The effect of extraction method on yield and antimicrobial activity of *Acronychia pedunculata*

Sanjeewa K. Rodrigo[a]

[a]The Open University of Sri Lanka

Abstract:
Acronychia pedunculata (Rutaceae) is often used in traditional medicine for treating various skin diseases. Previous studies have shown significant antibacterial activity of the plant against methicillin-sensitive and methicillin-resistant strains of Staphylococcus aureus (MSSA and MRSA). In the present study, methanolic extracts of different plant parts (Stem-bark, leaves, root, fruits) prepared by two extraction methods, Soxhlet and ultrasound-assisted solvent extraction (UASE), were examined for antimicrobial activity. The screening of the plant extracts for their *in vitro* antibacterial activity against standard *S. aureus* (ATCC 5923) and two MSSA, five MRSA strains was carried out using agar well diffusion method. Antifungal activity was determined against the fungus *C. cladosporioides* using the TLC bioautography method. Yields of extracts varied with the plant part and the extraction method. For *Acronychia pedunculata*, the Soxhlet method afforded higher yield for the leaf, stem-bark, and fruit/seed extracts than the sonicator method, while the root gave higher yields from the sonicator method. Antimicrobial activity varied depending on the plant part and the extraction method.

Keywords: Soxhlet extraction, sonication, *Acronychia pedunculata,* antimicrobial activity

1. Introduction

Investigating natural products for their medicinal potential has been a long-standing practice. These compounds, derived from various sources in nature, have proven valuable in developing therapeutic medications. It is worth noting that only a small percentage of natural sources have undergone thorough exploration for their medicinal properties. The chemical uniqueness associated with these products surpasses that of other sources. Exploring bioactive compounds from natural origins can lead to several possibilities: identifying known compounds with known or unknown activity as well as discovering previously unidentified compounds and their activities [1].

Phytochemical processing techniques involve maceration and Soxhlet extraction, which isolate specific chemical entities for characterization. These methods concentrate the bioactive compounds from plants, including polyphenols that exhibit antimicrobial, antioxidant, anti-inflammatory, and antiviral effects [2, 3]. Extraction is a fundamental step in phytochemical

Corresponding Author: srodr@ou.ac.lk

processing, which involves the separation of compounds of interest from natural sources like plant materials using solvents or other suitable methods. The choice of extraction method depends on the properties of the compounds being targeted and the nature of the source material. Most commonly used conventional extraction techniques include maceration, percolation, infusion, decoction, and hot continuous extraction like the use of Soxhlet extractor. Recently, alternative methods like ultrasound-assisted solvent extraction (UASE), microwave-assisted solvent extraction (MASE), and supercritical fluid extractions (SFE) have gained increasing interest during the last three decades [4]. Each extraction method has its advantages and is chosen based on factors such as the type of compounds being targeted, the properties of the plant material, efficiency, cost, and safety considerations. Once extracted, these bioactive compounds can undergo further analysis, purification, and formulation to create products with enhanced potency and efficacy for various applications. However, the yield and the bioactivities of the extracts obtained with different extraction methods have been reported to vary in several studies [5].

This study aims to compare the yield and level of antimicrobial activity of several methanolic extracts of *Acronychia pedunculata* (Sin: Ankenda) obtained using two extraction methods, soxhlet extraction and UASE. *A. pedunculata* is used in ayurvedic herbal preparations to treat various skin diseases [1]. In a recent study, the extracts of *A. pedunculata* prepared by Soxhlet apparatus have exhibited varying degrees of antibacterial activity against methicillin-sensitive and -resistant strains of Staphylococcus aureus (MSSA and MRSA), *Enterococcus faecalis* and *Pseudomonas aeruginosa* [6]. The steam distillate of *A. pedunculata* has shown antimicrobial and aphicidal activity [7]. Both Soxhlet and sonicator methods are used to prepare extracts from plants. However, the extraction method may affect the yield as well as the bioactivity of the extracts. In this study, we have compared the two extraction methods in terms of yield and bioactivities of *A. pedunculata*

2. Literature Review

A. pedunculata (L.) Miq. (Rutaceae) is a small, straight, stemmed, evergreen tree with a pale smooth bark and glabrous branches. While we see only this species in Sri Lanka, 42 species of the Genus, thrive in India to Southwestern China, Taiwan, throughout Malaysia, to Southeastern Australia and New Caledonia. This can be found in the moist region, from sea level up to 1600 m. Greenish-white colored flowers occur from February to April [8]. The roots, stems, leaves, and fruits of *A. pedunculata* are used in folk medicine for treating diarrhoea, tussis, asthma,

ulcers, itchy skin, scales, pain, and rheumatism [5]. Pulps or roots are used to treat gastric pain and hernial pain. Anorexia and indigestion are treated with a decoction of fruits. Using leaves, a decoction is made to treat colds and coughs. This plant, together with *Ligusticum wallichii* (Franch.), *Carthamus tinctorius* (L.), and *Salvia miltiorrhiza* (Bg.) is used to make a "coronary mixture" for the treatment of coronary heart disease [1].

Acronychia pedunculata Steam distillate of leaves has shown significant antibacterial activity against Staphylococcus aureus and mortality of Aphids, *Aphis craccivora Koch* [9]. Methanol extract of the stem and root bark has exhibited considerable cytotoxicity in the human KB tissue (Human epithelial carcinoma cells) culture assay [10], antiplasmodial activity, and brine shrimp toxicity. The CH_2Cl_2 stem bark extract has shown inhibition of cyclooxygenase-2 (COX-2). Terpenes, furanoqunoline alkaloids, coumarins, and some phenylethanones have been isolated from this plant [1]. Some bioactive compounds have been isolated from *A. pedunculata*, which include, acrovestone [7] (cytotoxic) [7], 7-acetyl-4,6-dihydroxy-5-(3-methyl-2-butyl)-2-(1-methyl-ethyl) benzofuran [10] (larvaecidal) [11], acrovestenol (COX-2 inhibition) [1].

3. Methodology

Plant materials were extracted with distilled methanol. Analytical TLC was carried out with 0.1 mm thick 60F Kieselgel G. Merck plates. UV active spots were located with 365 nm, and 254 nm tubes of a Bio block UV lamp.

3.1 Collection of plant material

Four parts of *A. pedunculata* were collected for this study. Bark samples of *Acronychia pedunculata were* collected from a tree located in the Nagolla area, Matale and the leaves, roots, and fruits were collected from a tree grown in Ratemulla, Uduwela area in Sri Lanka. Care was taken to avoid the collection of plant material contaminated with microorganisms like lichens, and fungus. Plant materials were identified by comparing them with those at the National Herbarium, Royal Botanical Garden, Peradeniya.

3.2 Preparation of the plant material for extraction

Each plant material was dried under mild sunlight inside the laboratory to a constant weight. Each material, cut into small pieces manually, was used in the Soxhlet extraction (SE). Materials, ground to a powder with a home-used grinder, were used in Ultrasound-assisted solvent extraction (UASE).

3.3 Extraction methods

3.3.1 Ultrasound-assisted solvent extraction (UASE)

A glass beaker, loaded with ground plant material and methanol (**Table 1**), and covered with aluminum foil, was placed inside the sonicator, which was partially filled with water. The contents were sonicated twice, for half an hour each time. After each sonication, the mixture was filtered through cotton wool, and the filtrate was collected and evaporated under reduced pressure using a rotary evaporator.

Table 1. Extraction conditions of the sonicator method applied for plants *A. pedunculata*

Plant part	Weight of dried plant material/g	Volume of methanol /mL
Root	25	150x2
Leaves	50	200x2
Stem-bark	50	150x2
Fruits	50	175x2

3.3.2 Soxhlet extraction

The dried and ground plant materials were Soxhlet extracted with methanol. Volume of methanol and duration of extraction are given in Table 2. In each extraction before terminating, TLCs of the bulk in the flask and the extractive coming out of the Soxhlet which was almost colourless, were recorded and compared to confirm the absence of new compounds in the colorless extractive.

Table 2. Extraction conditions of the Soxhlet method applied for plants *A. pedunculata*

Plant part	Weight of dried plant material/g	Volume of methanol /mL	Duration of extraction /h
Root	100	700	18
*Leaves	50	(1)700	12.5
		(2)700	12.5
*Stem-bark	50	(1)300	8.5
		(2)300	10
Fruit	50	350	24

3.4 Bioassays

3.4.1 Antibacterial activity

Each crude extract from both extraction methods was tested for antibacterial activity against methicillin-sensitive (MSSA) and –resistance (MRSA) *Staphylococcus aureus.*

3.4.1.1 Antibiotic sensitivity test (ABST) using agar well method

Sterilized Muller-Hinton Agar (MHA) (20 mL) at 50 ºC was poured into sterile Petri dishes and allowed to set on a flat surface. The MHA plates were inoculated with the bacterial suspension (2 mL). The extra amount of the suspension was removed, and the plates were allowed to dry inside an oven at 44 ºC for 15 minutes. Five wells were cut in the MHA plate using a cork borer. Hot sterilized MHA (one drop) was added to each well and allowed to set. 1000 ppm solutions of each extract were prepared. Each solution was added to wells so that one plate contained the four extractives of a plant. Extract-free solvent (DMSO or methanol) wells served as controls. Then, the plates were incubated at 37 ºC for 24 hours. Inhibition zone diameter was measured along five different axes, and averaged [12].

3.4.2 Antifungal activity

Each crude extract from both extraction methods was tested for antifungal activity against *Cladosporium cladosporioides.*

3.4.2.1 TLC bioautography of crude methanol extracts

Each extract was dissolved in MeOH and spotted on three TLC plates, and the plates were developed with the following three solvent systems.

a. 100% $CHCl_3$ (for low polar compounds)
b. 15% MeOH/ $CHCl_3$ (for medium polar compounds)
c. 7:3:1($CHCl_3$: MeOH: H_2O) (for high polar compounds)

Plates were air dried overnight and sprayed with a suspension of conidia scraped from a seven-day old culture of *C. cladosporioides* in Czapeck-dox nutrient solution (CNS). TLC plates were incubated in a moisture chamber for 48 hours. The inhibition area appeared white against a background of green mycelia of *C. cladosporioides* [13].

Statistical analysis

Data were expressed as mean ± standard deviation (S. D). Statistical analysis involved a one-way analysis of variance (ANOVA). A value of P less than 0.05 ($p < 0.05$) was considered statistically significant.

4. Results and Discussion

Biologically active compounds are typically found in low concentrations within plants or any other natural source. An extraction method should give high yields of extracts while minimizing alterations to the functional properties of the compounds in the extract [14]. Numerous studies have documented differences in the biological effects of extracts obtained through various extraction methods. Hence, it is imperative to carefully choose the appropriate extraction technique [15, 16]. The effectiveness of solvents in conventional extraction like Soxhlet is determined by factors such as the compound's solubility, mass transfer kinetics, and the interaction between solute and matrix. This also affects heat and mass diffusion rates during the process. The process of UASE involves the use of high-frequency sound waves and specific solvents to extract desired compounds from a variety of substances. This method has gained popularity in recent years due to its ability to improve extraction efficiency, reduce extraction time, and minimize the use of toxic solvents. Ultrasound changes the physical and chemical characteristics of materials as sound waves affect plant cell walls, resulting in the release of extractable compounds and improved solvent transport into plant cells [17].

4.1 Comparison of the two extraction methods: Soxhlet and UASE

Different parts of *Acronychia pedunculata* were extracted to methanol using Soxhlet and sonicator methods. First the percentage yields were compared, and the percentage yields corresponding to the two methods are summarized in Table 3. Extraction yield (mass of extract/mass of dry matter) was used as an indicator of the effects of the extraction conditions.

Table 3. Yields of extracts prepared by Soxhlet and UASE methods

Plant part	Percentage yield %	
	Soxhlet	**UASE**
Root	13.6	31.0
Leaves	26.5	9.2
Stem-bark	36.7	8.4
Fruits	21.0	7.0

As summarized in Table 3, yields of extracts vary with the type of plant material and also with the extraction method. Soxhlet method afforded higher yields of the leaf, stem-bark, and fruit extracts than the sonicator method. The roots, however gave a higher yield with the UASE method. This clearly shows that the extraction method has an impact on the extractability of compounds from the different plant parts. The Soxhlet technique resulted in the greatest yields across various plant parts, possibly because heat is utilized during Soxhlet extraction, facilitating the diffusion of solvents into materials with comparable polarities.

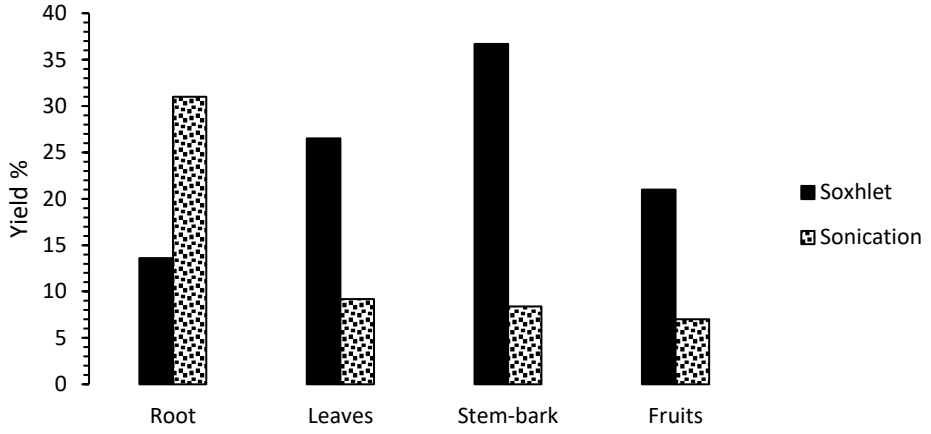

Figure 1. The yield of *A. pedunculata* extracts from the three extraction methods

4.2 Antibiotic sensitivity test

4.2.1 Antibacterial activity of crude extracts of *Acronychia pedunculata*

The extracts of *A. pedunculata* prepared employing the sonicator and Soxhlet methods (Table 3) were separately examined for their antibacterial activity against three strains of methicillin-sensitive *Staphylococcus aureus* and (MSSA) and four strains of methicillin-resistant *Staphylococcus aureus* (MRSA) using agar well method (Figure 2).

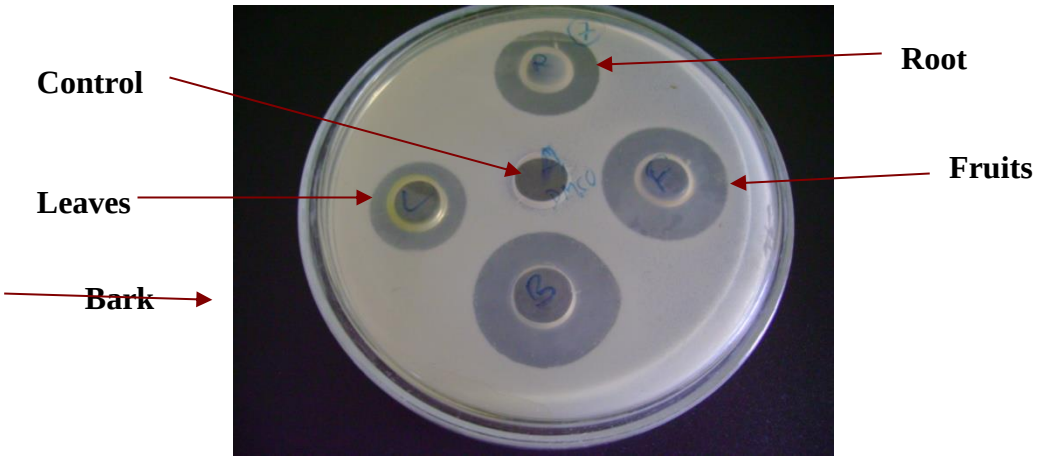

Figure 2. Antibiotic sensitivity test of Soxhlet extracts of *A. pedunculata* using agar well method

The diameters of the inhibition zones in the agar plates corresponding to *A. pedunculata* sonicator extracts are given in Table 4 and Figure 3.

Table 4. Inhibition zone diameters in agar plates of *S. aureus* corresponding to extracts (1000 ppm) of *A. pedunculata* prepared by sonicator and Soxhlet methods

Bacterial strain	Root		Leaves		Stem-bark		Fruits	
	Soxhlet	Sonication	Soxhlet	Sonication	Soxhlet	Sonication	Soxhlet	Sonication
Standard ATCC 5923	24.70±0.71	29.84±0.26	24.30±1.16	30.12±0.58	31.08± 0.77	28.56±0.83	27.81±0.73	25.00±1.16
1 (MSSA)	23.14±0.49	28.14±0.93	*22.32± 0.54	29.30±0.84	24.22± 0.45	*24.05±1.43	25.58± 0.59	21.24±1.23
2 (MSSA)	24.12±0.54	30.00±0.32	23.28±0.37	*31.35±081	*30.26±0.65	26.56±1.56	28.04±0.62	24.20±0.47
3 (MSSA)	24.94± 0.68	29.20±0.57	*22.57±0.59	29.80±0.47	30.63± 0.64	27.84±0.50	*29.57±0.74	24.72±0.48
4 (MRSA)	23.98±0.64	29.76±0.48	23.12± 0.30	30.04±0.84	29.52±0.76	27.16±0.86	30.78± 0.33	24.84±0.73
5 (MRSA)	24.14± 0.31	**29.93±0.46	*24.08±0.403	30.40±0.58	29.92± 0.228	25.52±0.46	29.85±0.72	21.80±0.83*
6 (MRSA)	24.58± 0.47	29.32±0.84	24.39±0.35	29.72±0.66	28.68±0.63	27.08±0.46	29.4±0.39	24.76±0.36
7 (MRSA)	24.17±0.29	29.92±0.36	23.86±0.85	30.08±0.54	30.22± 0.71	27.44±0.33	27.70± 0.66	25.20±0.40

† Average of five readings ± standard deviation, unless stated otherwise

*Average of four readings, **Average of three readings

#Inhibition zone diameters of controls - MeOH (13 mm), DMSO (14 mm)

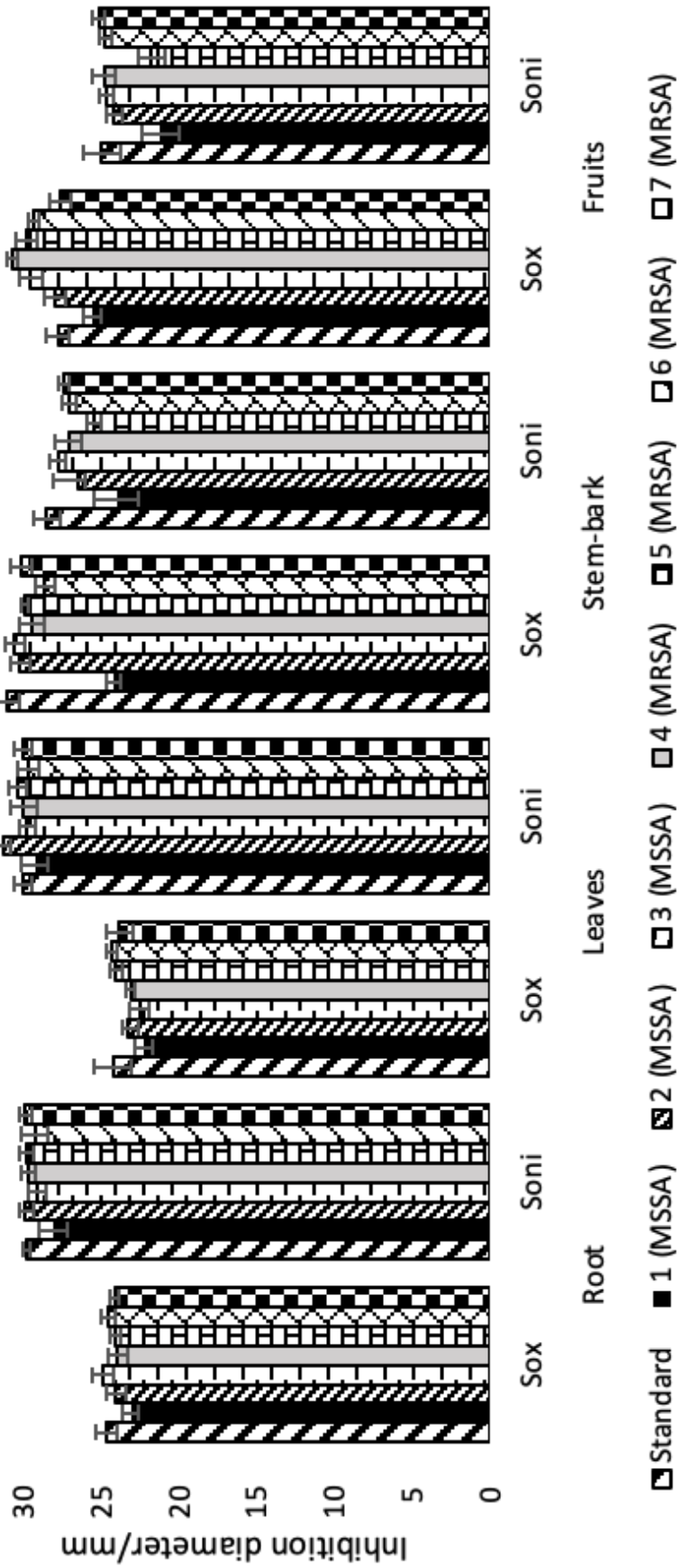

Figure 3. Graphical representation of inhibition zones of *A. pedunculata* extracts against *S. aureus*.

Statistical analysis

Inhibition zone diameters of extracts of both sonicator and Soxhlet extraction methods, were compared using "2-sample *t*-test" (Using Minitab 14.0). According to "2-sample *t*-test" the difference in antibacterial activities of two types of extracts (Soxhlet and sonicator) is statistically significant when $p<0.05$ at 95% confidence limit. Statistical analysis showed the inhibition zones corresponding to the sonicator extracts of *A. pedunculata* root and leaves were significantly ($p<0.05$) greater than those corresponding to the Soxhlet method. Inhibition zones corresponding to the Soxhlet extracts of *A. pedunculata* bark and fruits were significantly ($p<0.05$) greater than those corresponding to the sonicator method.

4.3 Antifungal activity

4.3.1 Antifungal activity of crude extracts of *Acronychia pedunculata*

The extracts of *A. pedunculata* prepared employing the sonicator and Soxhlet methods were separately examined for their antifungal activity against *Cladosporium cladosporioides* using TLC bioautography. Inhibition areas appeared as white coloured against the background of green mycelia of *C. cladosporioides* in TLCs of Soxhlet and sonicator extracts of *A. pedunculata* (Figure 4)

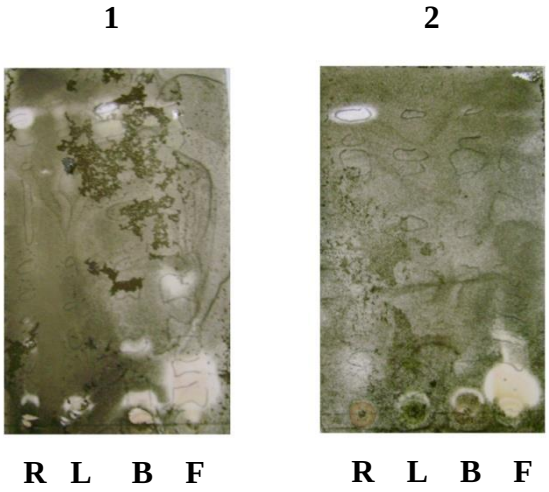

Figure 4. Results of TLC bioassay of *A. pedunculata* extracts (TLC developed using 100% $CHCl_3$), Key: 1=Soxhlet extracts, 2=Sonicator extracts, R=root, L=leaves, B=bark, F=fruit.

Each extract of *A. pedunculata* contains antifungal compounds. *A. pedunculata* Soxhlet extracts contain more antifungal compounds than sonicator extracts.

5. Conclusion

The findings confirmed that the extraction method affects the yield and bioactivity of extracts. Yields of extracts varied with the plant part and the extraction method. For *Acronychia pedunculata*, the Soxhlet method afforded higher yield for the leaf, stem-bark, and fruit/seed extracts than the sonicator method, while the root gave higher yields from the sonicator method. Antimicrobial activity varied depending on the plant part and the extraction method.

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