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RESEARCH ARTICLE

An Investigation into the Phytochemical Content, Antibacterial Effect, and Antioxidant Capacity of the Ethanol Extract of *Salacca wallichiana* Mart. Peels

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ABSTRACT

Objective: *Salacca wallichiana* Mart. is a prominent fruit-bearing tree distributed in Southeast Asia and used in treating many diseases and folk remedies. Thus far, only phytochemical composition-related research has been carried out on this plant, while the other bioactivities retarding its applicability in orthodox medicine have been ignored. Screening for the various bioeffects is needed to verify the authenticity of the medicinal activities of plants.

Materials and Methods: Following Ciulei separation, the phytochemical contents of the fruit peel extracts were determined. The antioxidant effect was evaluated by performing the free radical scavenging and potassium ferricyanide-reducing antioxidant power assays. Agar diffusion and broth dilution methods were used to ascertain the antibacterial capacity, and then the minimal inhibitory concentration (MICs) and MBCs were calculated.

Results: The results illustrated a robust free radical scavenging but a weak reducing activity. The MIC against Gram-positive bacteria was <4 mg/mL. The phytochemical composition included tannins and flavonoids, cardiac glycosides, organic acids, and reducing sugars.

Conclusion: The extracts of *S. wallichiana* peels demonstrated a potential antioxidant activity along with lethality against Gram-positive bacteria, which was attributed to the diversity in the contents of secondary metabolites.

Keywords: *Salacca wallichiana*, antioxidant, phytochemicals, antibacterial.

INTRODUCTION

According to ancient Babylonian records, herbs have been used for treating human diseases in the east as far back as 60,000 years ago.[1] Evidence exists for the identification and use of medicinal plants for treating many remedies throughout the history of medicine.[2] The lack of scientific experiment-based evidence is disadvantageous for folk medicine compared to Western medicine. Scientific development has enormously facilitated research on plant-based pharmaceutical products, providing scientific evidence leading to their acceptance and application.[3] Many healthcare and pharmaceutical products derived from plant extracts demonstrated diverse medical applications.[4] Secondary compounds in plants have a great potential for medicinal applications because of their antioxidant, anticancer, and antimicrobial bioactivities.[5,6]

The genus Salacca comprises ~ 20 species, mainly distributed in tropical areas such as Southeast Asia and the east of the Himalayas. *Salacca wallichiana* Mart. grows in many Southeast Asian countries, including Thailand, Vietnam, Malaysia, and Indonesia.[7,8] This plant has many practical applications, such as food, wood, and medicines.[7–9] Contrary to *S. zalacca, S. wallichiana* has not been widely researched, with limited studies and no reports on its bioactivity. Thus, this research aimed to screen the phytochemical composition and evaluate the effects of the peel extracts concerning antioxidant capacity and antibacterial ability through experiments, including qualitative chemical reactions, free radical scavenging, reducing power, disk diffusion, and broth dilution assays.

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MATERIALS AND METHODS

Plant Materials and Sample Preparation

The fruits of *Salacca wallichiana* Mart. trees in An Giang province, Vietnam, were harvested (voucher number AG-2018-0150). The fruit peels were collected, washed twice, and oven-dried thoroughly at 40°C. The dried sample was ground into a fine powder, added with ethanol at a ratio of 1:10 (w/v), and extracted for seven days.[10] The crude *S. wallichiana* extract (SWE) was obtained by filtering and rotary evaporating. The crude extract was weighed and dissolved in DMSO to obtain a stock solution of 200 mg/mL, which was stored at -20°C until use.

Phytochemical Detection

The chemical composition of the extract was screened using the method described by Cuilei (1993).[11,12] The SWE was separated into three fractions with different polarities: water, ethanol, and diethyl ether, and the secondary metabolites of each fraction were detected using various reagents and reactions, including Mayer and Wagner reagents for alkaloids;[13] Keller-Kiliani reaction for cardiac glycosides; Fehling's solution for reducing sugars;[14] reducing $FeCl_3$ reaction for polyphenols;[15] gelatin reaction for tannins;[16] foam formation for saponins;[17] and proanthocyanidins with an acidic solution.[18]

Free Radical Scavenging Evaluation

2,2-Diphenyl-1-picrylhydrazyl [DPPH] (Sigma-Aldrich, USA) free radical scavenging assay was performed with a slight modification of the method described by Hatano (1988) to determine the antioxidant effects of SWEs.[19,20] Different extract concentrations were supplemented with the same volume of 0.3 mM DPPH. After 30 min of incubation at 37°C, the OD_{517} of the mixture was recorded.

2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid [ABTS] (Sigma-Aldrich, USA) radical scavenging assay is based on forming ABTS cation radicals.[21,22] ABTS solution was prepared by adding a triple volume of 7.4 mM potassium peroxydisulfate [$K_2S_2O_8$] (Sigma-Aldrich, USA) into a specific volume of 2.6 mM ABTS for adjusting the reaction solution. It was dark-incubated for 16 h, and the OD_{734} was determined to be 1.00 ± 0.02. For the reaction, 750 µL of the active solution was added to 150 µL of the extract at varying concentrations, and the OD_{734} was ascertained.[20] Then, nonlinear regression equations were developed to determine the EC_{50}. Vitamin C (Sigma-Aldrich, USA) was used as a positive control, and DMSO as the negative control.

Reducing Power Assay

The potassium ferricyanide-reducing antioxidant power (PFRAP) assay was carried out using a modified method.[23] For this, 1 mL of the extract was diluted with 2.5 mL of phosphate-buffered saline [PBS, pH ± 6.6] (TBR Co., Vietnam) and then added with 2.5 mL of 1% potassium ferricyanide (Sigma-Aldrich, USA). After proper mixing and incubation for 20 min at 50°C, the mixture was added with 10% trichloroacetic acid (Sigma-Aldrich, USA), and the reaction was allowed for 10 min at room temperature. The supernatant was collected and diluted with the same volume of water. The solution was supplemented with a-nought-point-1-fold volume of 0.1% $FeCl_3$ (Sigma-Aldrich, USA), and then the OD700 of the teal color solution was determined.[20] The extract at 0 to 800 µg/mL was used for testing. Vitamin C was used as a positive control, and DMSO as the negative control.

Antibacterial Activity

The pathogenic bacteria selected to determine the antibacterial effects of the extracts included *Staphylococcus aureus* (ATCC 25923 and ATCC 6538), *Rhodococcus equi* (ATCC 6939), *Listeria monocytogenes* (ATCC 13932), *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 25933), *Shigella sonnei* (ATCC 9290), and *Salmonella enterica* (ATCC 14028). The bacteria were cultured in tryptic soy broth (Acumedia®, Neogen, USA) at 37°C overnight. Then, the agar-well diffusion method was used to determine the sensitivity of the bacterial species.[24] For this, a bacterial solution at 10^8 CFU/mL was prepared and spread out evenly on the surface of MHA [Mueller Hinton Agar] (HiMedia, India). After making 6-mm wells, 50 µL of the extract at different concentrations was loaded into the wells. The plate was incubated at 37°C for 20 h, and the inhibition zones were then measured. Ampicillin was used as the positive control.

Investigation of Antimicroorganism Lethality

The bacteria at a density of 10^5 CFU/mL were co-cultured overnight with varied concentrations of the extract in MHB [Mueller Hinton Broth] (HiMedia, India) in a 96-well plate. Then, 30 µL of 0.02% resazurin (Sigma-Aldrich, USA) was added to each well and incubated at 37°C for 30 min. The MIC (minimal inhibitory concentration) was defined as the lowest concentration of the SWE that maintained the blue color.[25] The bacteria at a concentration < MIC were seeded into TSA plates and incubated for ~ 20 h. The MBC (minimum bactericidal concentration) was identified as the least concentration of SWE at which the bacterial colony was nonostentatious.

Statistical Analysis

The experiments were conducted in triplicates. The data were expressed as mean±SD. The statistical analysis was performed using GraphPad Prism version 9.0.0. The Student's t-test and one-way ANOVA combined with Turkey Post-hoc tests were performed to ascertain the statistical significance of the differences at $P<0.05$.

RESULTS

The Diversity in the Secondary Metabolite Content of *S. wallichiana*

The medicinal powder was extracted thrice with ethanol. The average crude mass of 40 g of herbal powder was 2.70 ± 0.14 g, with an average extraction yield of 6.72 ± 0.23%. Triterpenoids were identified in the diethyl ether fraction. The ethanol and water fractions revealed a similar composition of polyphenols, including tannins and flavonoids, cardiac glycosides, organic acids, and reducing sugars (Table 1).

SWE Scavenged Free Radicals *In Vitro*

The antioxidant activity was evaluated using an *in vitro* model.[26] The antioxidant effects of the SWEs were investigated by conducting the DPPH and ABTS radical scavenging assays and the ferric-reducing power assay. The proportion of DPPH and ABTS radicals scavenged reached a peak at 100 µg/mL of SWEs with the DPPH assay (Figure 1) and 25 µg/mL with the ABTS assay (Figure 2). The nonlinear regression equation with $R^2 > 0.97$ for the free radical scavenging capacity of SWEs was established as "$Y = 100 \times (X^{1.306})/(17.431.306 + [X^{1.306}])$" and "$Y = 100$ x $(X^1.712)/(4.511.712 + [X^1.712])$" using DPPH and ABTS assays, respectively. The half maximal effective concentration (EC_{50}) was 17.43 ± 0.92 µg/mL for DPPH scavenging and 4.51 ± 0.21 µg/mL for ABTS scavenging.

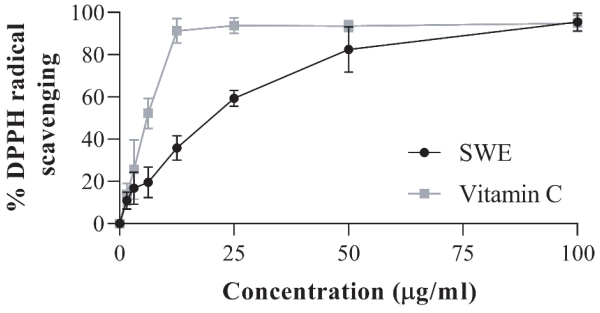

Figure 1. The free radical scavenging ability of the SWEs at 0 to 25 µg/mL as reflected by the proportion of DPPH neutralized. DMSO and Vitamin C were used as negative and positive controls, respectively.

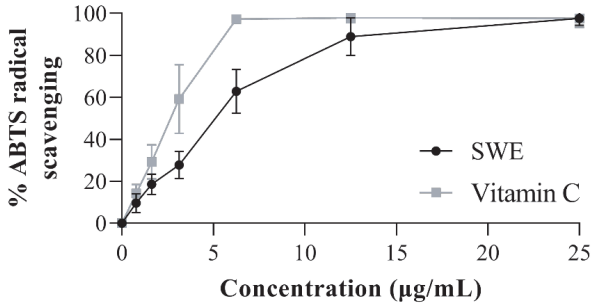

Figure 2. The free radical scavenging ability of the SWEs at 0 to 25 µg/mL was reflected in the proportion of ABTS neutralized. DMSO and vitamin C were used as negative and positive controls, respectively.

SWEs Reduced Fe^{3+} to Fe^{2+}

Fe^{3+} turns to Fe^{2+} under the action of a reducing agent, causing the solution to change from green to yellow. The reducing power of the SWEs was proportional to the concentrations of the extract (Figure 3), which was much lower compared to the control, indicating a limitation in participation in the direct reduction reaction.

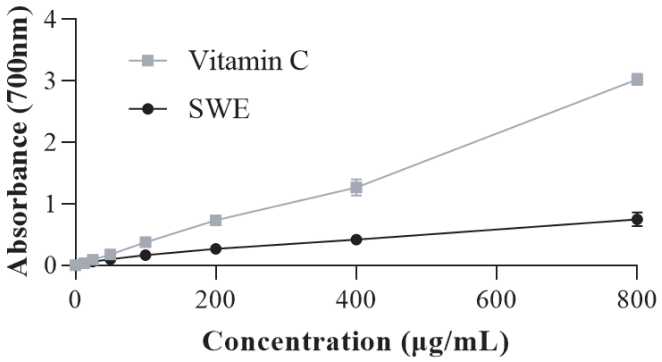

Figure 3. The Fe reduction reaction reflects the electron donor capacity of the SWEs. The reducing power of 0 to 800 µg/mL of the extracts was ascertained by measuring the reduction of Fe3+ to potassium ferricyanide. DMSO was used as blank. Vitamin C was used as a positive control.

SWEs Inhibited Gram-Positive Bacteria

The antibacterial activity of the different concentrations of the SWEs was ascertained using the disk diffusion assay against Gram-positive bacteria.[24] The inhibition zones were measured (Table 2 and Figures 4 A-D) and positively correlated with enhancing SWE concentrations of SWEs. *L. monocytogens* demonstrated the highest resistance to the SWEs.

Table 1. The phytochemical composition of the SWEs.

Phytochemicals	The diethyl eter fraction	The ethanol fraction	The water fraction
Triterpenoid	++	||	||
Alkaloid	-	-	-
Anthocyanosid	-	-	-
Proanthocyanidin	-	+++	+++
Polyphenol	|	+++	+++
Tannin	|	++	++
Saponin	|	+	+
Cardiac glycosid	|	+++	+++
Reducing sugar	|	+++	+++
Organic acid	|	++	++

Trace quantity (+); moderate quantity (++); appreciable quantity (+++); absence (-); no need for detection (||)

Table 2. The antimicrobial activity of the SWEs is indicated by the inhibition zones (mm).

Organism	The SWE concentration (mg/mL) – the inhibition zones (mm)				
	0	25	50	100	200
S. aureus ATCC 25923	0	8.44 ± 0.06	9.83 ± 0.01	11.03 ± 0.17	11.94 ± 0.28
S. aureus ATCC 6538	0	8.11 ± 0.06	9.83 ± 0.17	10.83 ± 0.26	11.78 ± 0.34
R. equi ATCC 6939	0	8.22 ± 0.11	9.50 ± 0.17	10.39 ± 0.34	11.28 ± 0.37
L. monocytogenes ATCC 13932	0	8.50 ± 0.01	8.72 ± 0.15	9.78 ± 0.15	10.64 ± 0.31
S. sonnei ATCC 9290	0	0	0	0	0
E. coli ATCC 25922	0	0	0	0	0
P. mirabilis ATCC 25933	0	0	0	0	0
S. enterica ATCC 14028	0	0	0	0	0

Table 3. The MIC and MBC values of the SWEs and ampicillin.

Organism	The SWE (mg/mL)		Ampicillin (µg/mL)	
	MIC	MBC	MIC	MBC
S.aureus ATCC 6538	1.875	>30	3.125	3.125
S.aureus ATCC 25923	1.875	>30	6.25	12.50
R.equi ATCC 6939	1.563	>30	3.125	6.25
L.monocytogenes ATCC 13932	3.125	>30	3.125	6.25

MIC Values of the SWE-Sensitive Bacteria

The MICs were 1.563–3.125 mg/mL, and MBCs were >30 mg/mL. Ampicillin was used as a positive control (Table 3 and Figure 4E).

DISCUSSION

The primary compounds, such as nucleic acids, proteins, carbohydrates, and lipids, play an essential role in plant sur-

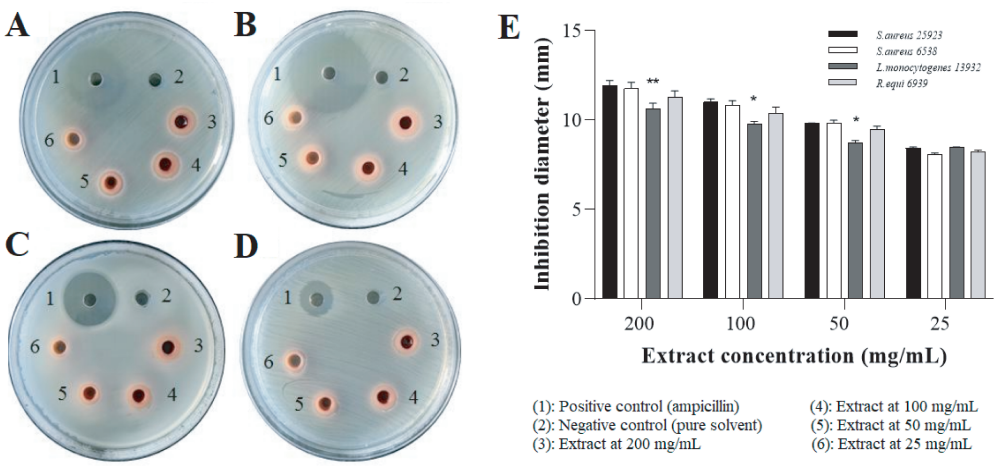

Figure 4. The antibacterial effects of the SWEs against Gram-positive bacteria were determined by the agar diffusion and broth dilution tests. The diameters of the zones of inhibition against *S. aureus* ATCC 25923 (A), *S. aureus* ATCC 6538 (B), *L. monocytogens* ATCC 13932 (C), and *R. equi* ATCC 6939 indicated as a graph (E). **P value < 0.002; ***P value < 0.001.

vival, unlike secondary metabolites, which vary from species to species.[27,28] Many function as cell signaling agents, luring insects or animals for pollination and seed dispersal, and in determining flower color.[28] Based on their chemical structure and functional groups, secondary metabolites directly interact with the cells via cell membranes, proteins including receptors, and nucleic acids, exhibiting a wide range of pharmacological properties such as robust antioxidant activity.[29] The discovery of secondary metabolites in plant extracts holds promise for applying their bioactivities, which can contribute to plant-based medical research.[30] The phytochemicals were extracted into three fractions based on polarity, using diethyl ether, ethanol, and water according to the previous method.[11,12] Based on the solubility of the types of phytochemicals, appropriate chemical reactions were used to detect their presence. Research on the chemical composition of *S. wallichiana* and related plants is limited. One report indicated the presence of flavonoids, alkaloids, tannins, terpenoids, and quinones in *Salacca edulis* fruits, while another detected quercetin and chlorogenic, gallic, cafeic acids in the extracts of *S. edulis* fruit peels.[31,32] Monogalactosyl diacylglycerols, triacylglycerols, β-sitosterol, β-sitosteryl-3β-glucopyranoside-6'-O-fatty acid esters, β-sitosterone, stigmasterol, linoleic acid, lupenone, and taraxerol were isolated from different parts of *S. wallichiana*.[7,9]

The antioxidant capacity of the plant extracts is contributed by the free electrons and atomic H donation by the secondary metabolites.[33] The term "free radical" was first suggested in the 1950s to refer to the oxidizing radicals formed during cellular activity and as the products of certain enzyme-catalyzed reactions.[34] A balance between antioxidants and free radicals in the body is always maintained.[35] However, when the contents of free radicals increase, it causes an imbalance, leading to oxidative-state-related stress.[36] The overaccumulation of free radicals causes various damages to cellular components such as membranes, DNA, and proteins, leading to diseases such as degenerative neurological, heart-related, cancer, and other diseases in humans.[37,38] Secondary metabolites such as polyphenols, flavonoids, and others have potent antioxidant capacity.[39] The EC_{50} values obtained by the two methods were statistically different, and the extracts were much more effective on ABTS radicals than on DPPH. Compared to ABTS radicals, DPPH radicals were less sensitive due to their inability to completely react with slow-acting antioxidant agents and their susceptibility to environmental conditions such as solvents and pH due to phenol oxidizing activity.[40] An EC_{50} of <10 µg/mL for the ABTS radicals suggested that the radical scavenging activity of the total peel extracts was highly efficient; hence, further research is needed.[41] The EC_{50} of the free radical scavenging ability of the peel- and seed-extracts of S. zalacca were 6.4 ± 1.8 µg/mL and 28.9 ± 4.7 µg/mL, which was quite similar to the results obtained in this study.[42] Moreover, the antioxidant capacity of SWEs was higher when compared to other well-known plants, such as green tea (C. sinensis), with EC_{50} values of 3.94 –6.67 µg/mL.[43] During a redox reaction, a reducing agent donates electrons.[44] Hence, an indirect method was used to assess the reducing properties of the extracts to evaluate their antioxidant activity.[45] Fe^{3+} are potent oxidizing agents that readily participate in redox reactions and are often used to qualitatively determine the reducing activities of phytochemicals.[46,47] The PFRAP assay is based on the reduction of Fe^{3+} in potassium ferricyanide to Fe^{2+} catalyzed by an antioxidant agent[23], which demonstrated the reducing ability of the extracts attributed to the presence of secondary metabolites.

The susceptibility of bacteria to treatment with SWEs was determined based on the diameter of the inhibition zones; with a broader diameter indicating an enhanced sensitivity. Phyto-

chemicals possess a potent antibacterial activity, which holds enhanced promise for the applicability of SWEs due to the detection of diverse chemical constituents.[48] This study showed that SWEs were toxic to Gram-positive bacteria. The outer membrane is unique in Gram-negative bacteria, forming a protective barrier that prevents the entry and permeation of hydrophilic agents. Therefore, drug resistance in Gram-negative bacteria is higher than in Gram-positive bacteria.[49] The agar diffusion assay indicated that the species were susceptible to SWEs further used for the broth dilution assay. The broth dilution method was the secondary screening assay that allowed for the determination of the MICs and MBCs. The bacteria cultured in MHB were exposed to the extracts added with 0.02% aniline resazurin and further subcultured on MHA to determine the MBC. Growth inhibition and lethality against bacteria indicated the antimicrobial activity of the SWEs. *S. zalacca* showed moderate antibacterial activity with inhibitory zone diameters of 7.31 ± 0.82 mm and 7.17 ± 0.86 mm for *S. aureus* and *S. typhi*, respectively[50] and also on the Gram-negative bacteria, *E. coli*, with an inhibition zone of 5.96 ± 1.45 to 8.13 ± 0.40 mm.[51,52]

CONCLUSION

The phytochemical screening revealed that the SWEs contained terpenoids and polyphenols, including tannins and flavonoids, cardiac glycosides, organic acids, and reducing sugars, contributing to potent antioxidant and antibacterial effects. The antibacterial effects of SWEs were observed against Gram-positive bacteria with MIC values <4 mg/mL.

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