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Antibacterial and antioxidant activities of methanolic leaf extracts of some *Annonaceous* plants found in Nakhon Si Thammarat, Thailand

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ABSTRACT

The leaves of 20 species belonging to the Annonaceae family were collected and extracted using methanol to obtain scientific data to evaluate their potential use as new sources of medicinal agents. Methanolic leaf extracts 1–20 were subjected to phytochemical screening and a biological activity assay. The multipharmacophore of the natural products with the synergistic action was remarked as the target. The antimicrobial activity assay was done in parallel with the antioxidant activity test. Among these 20 extracts, extracts 1 and 4 exhibited the inhibition of the growth of all the test microbes. Flavonoids and tannins seem to be the main components of this action. Dramatic results indicate that extract 1 was the most active as an antioxidant. This research was the first report on the biological activities of extract 20. Extract 20 exhibited bactericidal activity against *Enterococcus faecalis* at 1,000 µg/ml. Terpenes might be the main component of this action. This result was related to the antimicrobial activity of extract 2 which contains terpenes and exhibited high antimicrobial activities against *Staphylococcus aureus* and *E. faecalis* (minimum inhibition concentration, MIC = 250 µg/ml). These data indicated a promising potential for *Annonaceous* plants to be used as sources of naturally occurring medicinal agents.

INTRODUCTION

On account of its biodiversity, the Nakhon Si Thammarat area in Thailand is an important reservoir of natural resources. Nakhon Si Thammarat comprises several environmental habitats, ranging from the Khao Luang National Park to the Gulf of Thailand, which provide a correspondingly wide variety of natural resources (Sutthipibul, 2013). In traditional Thai medicine, some *Annonaceous* plants have been used to cure pathological conditions, including snakebites, diarrhea, dysentery, arthritic pain, rheumatism, and neuralgia, as well as for analgesic, astringent, and weight-loss purposes (Choudhary *et al*., 2015). Recently, some *Annonaceous* plants have even been used to treat cancer and provide other health benefits. However, safety profiles and scientific data for these plants are needed to consolidate their promising potential use. For example, multidrug-resistant bacteria represent a severe problem in the global healthcare system. Opportunistic infections by such bacteria cause severe human health issues and worldwide economic problems as affected patients require special treatment and long hospital stays for the administration of drugs with higher antibacterial activity and intensive health interventions (Aslam *et al*., 2018). Many other kinds of microorganisms also affect human health and create public health problems, such as *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, and *Acinetobacter baumannii*, which can be found in contaminated food and cause health conditions including food poisoning, gastritis, colitis, and severe sepsis (Castellanos *et al*., 2019). *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are pathogens that also cause opportunistic infections, whereby the latter is often drug-resistant (Azimi *et al*., 2019). *Candida albicans* is a yeast that causes infections, especially in patients with weakened immune systems (Mayer *et al*., 2013). Degenerative diseases associated with free radicals, including atherosclerosis, cancer, inflammatory joint disease, asthma, diabetes, senile dementia, and degenerative eye disease, also represent serious problems for the general population

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(Furman *et al*., 2019). Therefore, it is important and urgent for phytochemists to investigate bioactive compounds from, for example, *Annonaceous* plants with respect to their potential to be used against pathogens and to protect human health from degenerative diseases. In this study, we have collected several samples of *Annonaceous* plants during excursions to various areas of Nakhon Si Thammarat. The antimicrobial activities of the leaves of the samples were determined using a broth microdilution assay, and their antioxidant properties were screened using a 2,2-diphenyl-1-picrylhydrazyl- (DPPH-)-based free radical scavenging assay.

MATERIALS AND METHODS

The leaves of 20 species of *Annonaceous* plants were collected in the Sichon, Thasala, and Muang districts of Nakhon Si Thammarat (Thailand) between June and August 2021. Voucher specimens were identified and deposited at Walailak Botanical Park, Walailak University. All samples were dried using a hot-air oven at 50°C and then ground into a powder using a mill. The samples were extracted in refluxing methanol (samples 1–10: 3 × 4 l; samples 11–20: 3 × 0.5 l; 3 hours). The combined methanolic extracts were filtered before all volatiles were removed from the filtrate under reduced pressure to yield the crude methanolic extract.

Phytochemical screening

Test for alkaloids

A small amount of each crude methanolic extract was individually boiled with a 10% sulfuric acid solution (5 ml) for 10 minutes. The filtrates were treated with Dragendorff's reagent. If a sample contains alkaloids, an orange-red amorphous precipitate is formed during the reaction (Raal *et al*., 2020).

Test for flavonoids

A small amount of each methanolic leaf extract was individually dissolved in methanol (3 ml). Each methanol extract was treated with a few drops of ammonium hydroxide test solution (TS). If the sample contains flavones or flavonols, a yellow precipitate is formed and the solution will turn its color. In the case of flavanones, an orange-red solution is observed, while in the case of chalcones or aurones, the TS changes color to magenta (Hossain *et al*., 2013).

Test for tannins

A small amount of each crude methanol extract was individually boiled with distilled water (5 ml) before a saturated lead subacetate solution (1 ml) was added to 2 ml of the filtrate of each sample. The formation of a precipitate indicates the presence of tannins. Additionally, a small sample (2 ml) of the filtrate of each extract was treated with a few drops of a 1% ferric chloride solution. The formation of a greenish-black (turquoise) precipitate indicates the presence of condensed tannins, while the formation of a deep blue precipitate indicates the presence of hydrolyzable tannins (Heera *et al*., 2012).

Test for anthraquinones

Modified Borntrager's test – To a small sample (1 ml) of each methanol extract, 1 ml of a 5% ferric chloride solution and 1 ml of 10% sulfuric acid were added before the resulting solution was heated to ~90°C for 10 minutes in a water bath. After cooling, 1 ml of chloroform was added. The sample was shaken well, and the organic layer was separated. An equal volume of 10% sodium hydroxide solution was then added. The formation of a rose-pink/red precipitate at the ammonia layer indicates the presence of anthraquinones (Auwal *et al*., 2014).

Test for steroids and terpenoids

Liebermann -Burchard test – A small amount (1 ml) of the methanolic extract of each sample was dried in a porcelain dish. Then, chlorophylls were washed out using a small amount (1–2 ml) of chloroform before a few drops of acetic anhydride were added to the sample and mixed well. One drop of concentrated sulfuric acid was added next to the sample, and both fractions were allowed to mix. If a sample contains steroids, a green to blue-green color will emerge during the reaction. If the sample contains terpenoids, a purple-red or magenta color will emerge during the reaction (Mujeeb *et al*., 2014).

Test for coumarins

A small amount of each crude methanolic extract (1–2 g) was individually boiled with distilled water (5 ml). A small amount of the filtrate of each sample (2 ml) was treated with 1 ml of a 6 M sodium hydroxide solution. If a sample contains coumarins, the solution will turn yellow to dark yellow during the reaction (Stefanachi *et al*., 2018).

Test for saponin glycosides

A small amount of each crude methanolic extract was individually boiled with distilled water (5 ml). A small sample of the filtrate (2 ml) of each sample was shaken for 30 seconds. If a sample contains saponin glycosides, it exhibits stable honeycomb-shaped bubbles (El Hazzam *et al*., 2020).

Biological evaluation

Antioxidant assay

The DPPH-based free radical scavenging assay was chosen to evaluate the potential of the crude methanolic extracts to serve as antioxidants. We slightly modified the process for the preparation of the TS, positive control, and samples from the method described by Braca *et al*. (2001). *L*-ascorbic acid was used as a positive control. *L*-ascorbic acid (10 mg) was dissolved in methanol (10 ml) to prepare a 1,000 µg/ml solution, which was then diluted using the serial dilution method with the same solvent to give solutions of concentrations of 100, 10, 1, and 0.1 µg/ml. All samples were dissolved and diluted with methanol to give concentrations of 1,000, 100, 10, 1, and 0.1 µg/ml. About 100 µl of the positive control and the various concentrations of each sample were mixed with 100 µl of DPPH. About 100 µl of DPPH mixed with 100 µl of methanol was used as a control. About 200 µl methanol was used as the blank control. The blank sample was prepared by mixing 100 µl of the sample with 100 µl of methanol. All reactions were carried out within 30 minutes in the dark before the absorbance at 517 nm was measured using a microplate reader. All data were used for the calculation of $\%SC_{50}$ (Braca *et al*., 2001).

$$\% SC = \frac{[Ab_{control} - Ab_{control\ blank}] - [Ab_{sample} - Ab_{sample\ blank}]}{[Ab_{control} - Ab_{control\ blank}]} \times 100$$

Antimicrobial assay

The potentials of all the samples to serve as antimicrobial agents were measured using a broth microdilution assay to determine the minimum inhibition concentration (MIC) and minimum microbicidal concentration (MMC). Resazurin was used as the indicator. The test microbes were *E. coli* ATCC 25922 (Croxen *et al*., 2013), *S. aureus* ATCC 25923 (Tong *et al*., 2015), *P. aeruginosa* (Duraisingham *et al*., 2014), *E. faecalis* (Anderson *et al*., 2016), *A. baumannii* (Howard *et al*., 2012), *K. pneumoniae* (Paczosa *et al*., 2016), and *C. albicans* (Martins *et al*., 2014). *Pseudomonas aeruginosa*, *E. faecalis, A. baumannii, K. pneumoniae*, and *C. albicans* were collected and isolated from patients at Maharaj Nakhon Si Thammarat Hospital. The identified microbes were cultured on trypticase soy agar at 37°C, except for *C. albicans*, which was cultured on Sabouraud dextrose agar (SDA) at 25°C. A single colony of each of the bacteria was inoculated into 2X Mueller–Hinton broth (MHB), and *C. albicans* was inoculated into 2X Sabouraud dextrose broth. Before the assay, all microbes were incubated for 3 hours using the same conditions as previously described. All microbes were then diluted with a 0.85% sodium chloride solution using aseptic techniques to give concentrations equal to 0.5 on the McFarland scale. The bacteria were diluted 100 times with the same solution before the assay. All samples were prepared as solutions with dimethyl sulfoxide and 2X MHB to give a concentration of 1,000 µg/ml and diluted using the serial dilution method. About 10 µl of microbes was added to each concentration of each sample. All test solutions were mixed well and incubated at the same temperature for 24 hours. A resazurin solution was used as an indicator; 10 µl of the solution was added to each well and then incubated for 3 hours at the same temperature. In the presence of living microbes, resazurin irreversibly changes color from deep blue to pink, which is detectable via visual observation. The lowest concentration of each sample that inhibited the microbe was the MIC. The solutions in the wells for which the color of resazurin did not change were dropped on MHA (for bacteria) or SDA (for yeast and fungi) and then incubated for 3 hours at the same temperature. The lowest concentration where microbe growth did not occur is the MMC. Erythromycin and ketoconazole were used as positive controls for the bacteria and fungi, respectively (Balouiri *et al*., 2016).

RESULTS AND DISCUSSION

Twenty species of *Annonaceous* plants were collected from the Sichon (1–4, 6–9, and 19–20), Thasala (5, 10–13, and 16–17), and Muang (14–15 and 18) districts of Nakhon Si Thammarat (Thailand). Some Annonaceous plant pictures in this research were shown in Figure 1. Voucher specimens were identified and deposited at Walailak University. The leaves of the samples were cleaned and dried at 50°C. Each sample was milled into a powder, which was subsequently extracted with refluxing methanol (samples 1–10: 3 × 4 l; samples 11–20: 3 × 0.5 l). The combined methanol fractions were filtered before all volatiles were removed from the filtrate under reduced pressure to yield the crude methanolic extract. The percent yield of all samples is shown in Table 1.

The phytochemical screening of the methanolic leaf extracts was carried out using a variety of tests. Summary of the phytochemical test results of all samples is shown in Table 2. The detail of test results was presented in Supplementary Materials. Dragendorff's reagent was used for alkaloid testing. A small amount of each methanolic leaf extract was individually boiled with 10% H_2SO_4 (5 ml) in a water bath for 10 minutes. After filtration, the filtrates (1 ml) were treated with five drops of Dragendorff's reagent. An orange-red amorphous precipitate was observed for samples 3, 4, 9, 11, 14, and 15, which indicates the presence of alkaloids. We used Borntrager's test and a modified Borntrager's test to confirm the absence of anthraquinone in all samples. The Liebermann–Burchard test was modified for steroid and terpenoid testing. All samples were treated with chloroform to remove some chlorophylls. The obtained results indicated that samples 1–20 contained terpenoids and that samples 2, 3, 9, 10, and 12–20 also contained steroids. Raymond's reagent and Keddé's reagent were applied to test for the C-methylene of the lactone ring of acetogenins. These reagents have been used to detect the C-methylene of the unsaturated lactone ring of cardiac glycosides. In our investigation, a precipitate occurred in the reactions of samples 1–2, 5, 7–9, and 11–18, which suggests that these contain acetogenins (Akaike, 2001). The froth test (El Hazzam *et al*., 2020) was used to test for saponin glycosides. A small amount of each methanolic leaf extract was individually boiled with distilled water (5 ml). After filtration, the filtrates (2 ml) were shaken for 30 seconds and examined. Honeycomb-shaped bubbles that remained stable for more than 30 minutes after shaking indicate the presence of saponin glycosides. Saponin glycosides were observed for samples 1–3, 5–8, 11, 13–15, 17, and 19. The other filtrates were tested for tannins using 1% ferric chloride and saturated lead subacetate solutions. Methanolic leaf extracts 1–20 were found to contain condensed tannins based on the test using ammonium hydroxide TS, in which all samples changed color to red-orange and formed a yellow precipitate. These results confirm the presence of flavonoids in all the samples. To determine the presence of coumarins, 1 ml of each of the filtrates was treated with 1 ml of 6 M sodium hydroxide solution. Samples 1–12, 14, 16–18, and 20 turned yellow during this reaction, which confirms the presence of coumarins.

Antioxidant activity

The DPPH-based free radical scavenging assay was used to determine the antioxidant activity of the methanolic leaf extracts relative to that of *L*-ascorbic acid. The results suggest that sample 1 ($\%SC_{50} = 5.39 \pm 0.12$ µg/ml), sample 18 ($\%SC_{50} = 7.38 \pm 0.83$ µg/ml), and sample 2 ($\%SC_{50} = 11.55 \pm 0.82$ µg/ml) show the highest activity among the tested samples. The DPPH-based free-radical-scavenging assay result of all samples is shown in Table 3. Some calculations of the result were presented in Supplementary Materials.

Antimicrobial activity

The broth microdilution assay was applied to evaluate the antimicrobial activity of the methanolic leaf extracts. Resazurin was used as an indicator. Ampicillin and ketoconazole were used as positive controls for antibacterial and antifungal activity, respectively. The minimum concentration that inhibited the growth of the microbes is referred to as the MIC. The minimum concentration that killed the microbes is referred to as the MMC. A summary of the results is shown in Table 4.

The methanolic leaf extracts showed microbiostatic activity against the tested microbials. Samples 1–10 and 20 inhibited the growth of *S. aureus* at 250–500 µg/ml (sample 2

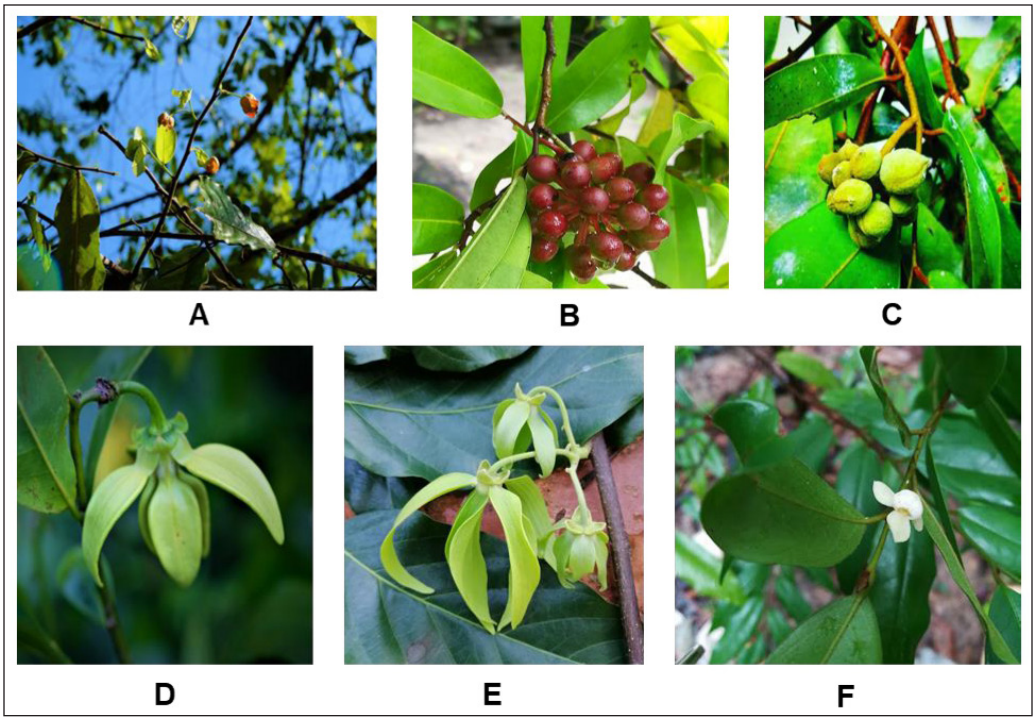

Figure 1. Some *Annonaceous* plants in this research; A (Sample 1)-*Miliusa lineata* (Flowers, leafs, and stem), B (Sample 2)-*Polyalthia suberosa* (Leafs and fruits), C (Sample 10)-*Rauwenhoffia siamensis* (fruits, leafs, and stem), D (Sample 16)-*Artabotrys hexapetalus (Flower), E (Sample 17)-Cananga odorata* (Flower, leafs, and stem), and F (Sample 20)-*Mitrephora chulabhorniana* (Flower, leafs, and stem). (Damthongdee *et al*., 2019; Pooma and Suddee, 2014)

Table 1. Methanolic leaf extracts of some *Annonaceous* plants in Nakhon Si Thammarat, Thailand (Sample 1–20). (Pooma and Suddee, 2014)

Sample	Plant name	Weight of the powder sample (g)	Weight of the dried MeOH extract (g)	%Yield (*w*/*w*)
1	*Miliusa lineata*	334.00	78.30	23.44
2	*Polyalthia suberosa*	280.00	50.91	18.18
3	*Anaxagorea javanica*	230.00	42.42	18.44
4	*Desmos chinensis*	311.42	58.18	18.68
5	*Uvaria rufa*	341.14	63.31	18.56
6	*Winitia cauliflora*	332.00	73.79	22.23
7	*Uvaria curtisii*	336.00	79.49	23.66
8	*Frisodielsia desmoids*	274.00	70.27	25.65
9	*Platymitra macrocarpa*	580.00	118.57	20.44
10	*Rauwenhoffia siamensis*	300.00	53.51	17.84
11	*Dasymaschalon blumei*	60.44	9.55	15.80
12	*Desmos cochinchinensis*	70.78	10.80	15.26
13	*Goniothalamus tapis*	66.70	11.81	17.71
14	*Alphonsea elliptica*	74.48	22.35	30.01
15	*Uvaria grandiflora*	71.63	15.03	20.98
16	*Artabotrys hexapetalus*	69.71	17.23	24.72
17	*Cananga odorata*	96.00	28.31	29.49
18	*Annona squamosa*	72.35	15.91	21.99
19	*Sageraea elliptica*	81.10	15.43	19.03
20	*Mitrephora chulabhorniana*	28.00	4.65	16.61

Table 2. Phytochemical screening of methanolic leaf extracts of some *Annonaceous* plants in Nakhon Si Thammarat, Thailand (Sample1–20). (Auwal *et al*., 2014; El Hazzam *et al*., 2020; Heera *et al*., 2012; Hossain *et al*., 2013; Raal *et al*., 2020; Stefanachi *et al*., 2018)

No.	Plant name	Alkaloids	Steroids	Terpenes	Flavonoids	Acetogenins	Saponins	Tannins
1	*Miliusa lineata*		●		●	●	●	●
2	*Polyalthia suberosa*		●	●	●	●	●	●
3	*Anaxagorea javanica*	●	●	●	●		●	●
4	*Desmos chinensis*	●	●		●			●
5	*Uvaria rufa*		●		●	●	●	●
6	*Winitia cauliflora*		●		●		●	●
7	*Uvaria curtisii*		●		●	●	●	●
8	*Frisodielsia desmoids*		●		●	●	●	●
9	*Platymitra macrocarpa*	●	●	●	●	●		●
10	*Rauwenhoffia siamensis*		●	●	●			●
11	*Dasymaschalon blumei*	●	●	●	●	●	●	●
12	*Desmos cochinchinensis*		●	●	●	●		●
13	*Goniothalamus tapis*		●	●	●	●	●	●
14	*Alphonsea elliptica*	●	●	●	●	●	●	●
15	*Uvaria grandiflora*	●	●	●	●	●	●	●
16	*Artabotrys hexapetalus*		●	●	●	●		●
17	*Cananga odorata*		●	●	●	●	●	●
18	*Annona squamosa*		●	●	●	●		●
19	*Sageraea elliptica*		●	●	●		●	●
20	*Mitrephora chulabhorniana*		●	●	●			●

● = availability of the compound in the leaf methanol extract.

was the most active; MIC = 250 µg/ml). Samples 1–10, 12, 13, and 16–20 showed inhibitory activity against *E. coli* (MIC = 250–500 µg/ml). Samples 1–10 showed inhibitory activity against *A. baumannii* (MIC = 125 µg/ml). Samples 1–10, 13, and 16–20 showed inhibitory activity against *P. aeruginosa* (MIC = 125–250 µg/ml), while samples 1–10, 13, 17, 19, and 20 showed inhibitory activity against *K. pneumoniae* (MIC = 250–500 µg/ml). Samples 1–10 showed inhibitory activity against *E. faecalis* (MIC = 250–500 µg/ml), whereas sample 20 showed strong activity against *E. faecalis* (MIC = 50 µg/ml and MMC = 1,000 µg/ml). Only samples 1 and 4 exhibited fungistatic activity against *C. albicans* (MIC = 125 µg/ml).

20 species of *Annonaceous* plants were collected during an excursion in Nakhon Si Thammarat (Thailand). Voucher specimens were prepared and deposited at Walailak University. Ten samples (1–4, 6–9, and 19–20) were collected from a limestone mountain in the Sichon district. Seven samples (5, 10–13, and 16–17) were collected from the Thasala district. Samples 5 and 10 are ubiquitous native plants all across Nakhon Si Thammarat. Three samples (14, 15, and 18) were collected from the Muang District of Nakhon Si Thammarat. The leaves of all samples were used to prepare methanolic leaf extracts. The average percentage yield (*w*/*w*) of the extracts was 20. Phytochemical screening of the methanolic leaf extracts was carried out using a variety of standard tests. The test for alkaloids was conducted using Dragendorff's reagent. The formation of an orange-red precipitate in the reaction indicates the presence of alkaloids, which occurred for samples 3, 4, 9, 11, 14, and 15. The amount of the orange-red precipitate that formed in samples 9, 11, and 14 was particularly high, which is

Table 3. Antioxidant activity of the methanolic leaf extracts of some *Annonaceous* plants in Nakhon Si Thammarat, Thailand (Extract 1–20) and L-ascorbic acid. (Akaike, 2001; Braca *et al*., 2001)

Sample	Plant name	%SC_{50} (µg/ml)
1	*Miliusa lineata*	5.39 ± 0.12
2	*Polyalthia suberosa*	11.55 ± 0.82
3	*Anaxagorea javanica*	74.42 ± 0.04
4	*Desmos chinensis*	23.14 ± 2.63
5	*Uvaria rufa*	18.91 ± 1.56
6	*Winitia cauliflora*	46.04 ± 1.97
7	*Uvaria curtisii*	33.01 ± 1.61
8	*Frisodielsia desmoids*	26.28 ± 1.31
9	*Platymitra macrocarpa*	36.03 ± 2.64
10	*Rauwenhoffia siamensis*	16.78 ± 2.57
11	*Dasymaschalon blumei*	28.21 ± 1.38
12	*Desmos cochinchinensis*	15.78 ± 0.87
13	*Goniothalamus tapis*	14.03 ± 1.55
14	*Alphonsea elliptica*	16.35 ± 0.13
15	*Uvaria grandiflora*	63.74 ± 2.46
16	*Artabotrys hexapetalus*	58.40 ± 2.46
17	*Cananga odorata*	22.99 ± 0.38
18	*Annona squamosa*	7.38 ± 0.83
19	*Sageraea elliptica*	39.57 ± 2.71
20	*Mitrephora chulabhorniana*	59.72 ± 2.03
21	L-ascorbic acid	3.73 ± 0.02

Table 4. Results of a broth microdilution assay to examine the antimicrobial activity of the methanolic leaf extracts of some *Annonaceous* plants in Nakhon Si Thammarat, Thailand (Sample 1–20) and the positive controls ampicillin (22) and ketoconazole (23).

Sample No.	Plant name / positive control	MIC/MMC; µg/ml (1–20), µM (22–23)						
		Staphylococcus aureus	***Escherichia coli***	***Acinetobacter baumannii***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***	***Enterococcus faecalis***	***Candida albicans***
1	*Miliusa lineata*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	125/ND
2	*Polyalthia suberosa*	250/NO	250/NO	125/NO	125/NO	250/NO	250/NO	NO/ND
3	*Anaxagorea javanica*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	NO/ND
4	*Desmos chinensis*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	125/ND
5	*Uvaria rufa*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	NO/ND
6	*Winitia cauliflora*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	NO/ND
7	*Uvaria curtisii*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	NO/ND
8	*Frisodielsia desmoids*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	NO/ND
9	*Platymitra macrocarpa*	500/NO	250/NO	125/NO	125/NO	250/NO	250/NO	NO/ND
10	*Rauwenhoffia siamensis*	500/NO	250/NO	125/NO	125/NO	250/NO	500/NO	NO/ND
11	*Dasymaschalon blumei*	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND
12	*Desmos cochinchinensis*	NO/ND	500/NO	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND
13	*Goniothalamus tapis*	NO/ND	500/NO	NO/ND	250/NO	500/NO	NO/ND	NO/ND
14	*Alphonsea elliptica*	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND
15	*Uvaria grandiflora*	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND	NO/ND
16	*Artabotrys hexapetalus*	NO/ND	500/NO	NO/ND	250/NO	NO/ND	NO/ND	NO/ND
17	*Cananga odorata*	NO/ND	500/NO	NO/ND	250/NO	500/NO	NO/ND	NO/ND
18	*Annona squamosa*	NO/ND	500/NO	NO/ND	250/NO	NO/ND	NO/ND	NO/ND
19	*Sageraea elliptica*	NO/ND	500/NO	NO/ND	250/NO	500/NO	NO/ND	NO/ND
20	*Mitrephora chulabhorniana*	500/NO	500/NO	NO/ND	250/NO	500/NO	50/1,000	NO/ND
22	Ampicillin	0.01/0.01	0.98/0.98	125/125	125/1,000	125/125	0.06/0.06	-
23	Ketoconazole	-	-	-	-	-	-	125/500

probably related to the amount of alkaloids present in the sample. The Liebermann -Burchard test was used to gauge the presence of steroids and terpenoids. The blue-green color observed for samples 2, 3, and 9–20 indicated that these samples contained steroids. The purple-red or magenta color observed in all samples indicated that all the samples contained terpenoids. To screen for acetogenins, we applied reagents used to test for the C-methylene of the unsaturated lactone ring in cardiac glycosides, that is, Raymond's reagent and Keddé's reagent. The color of the methanolic leaf extracts did not change, albeit a precipitate was formed in the reactions of samples 1, 2, 5, 7–9, and 11–18, indicating that these contain acetogenins. The froth test was used to screen for saponin glycosides, which were found in samples 1–3, 5–8, 11, 13–15, 17, and 19; samples 6 and 11 contained especially high amounts of saponin glycoside, as evident from the high amount of stable honeycomb-shaped bubbles. All samples were treated with a saturated lead subacetate solution to form a precipitate, indicating that all samples contained tannins. To identify the kind of tannins, a 1% ferric chloride solution was used. All samples formed a turquoise/dark-green precipitate, confirming the presence of condensed tannins. These results are all related to the presence of flavonoids. All samples (1–20) reacted with ammonium hydroxide TS to give a red-orange solution and a yellow precipitate. The condensed tannins have a core flavonoid structure. The water-soluble part of the methanolic leaf extracts was also tested for coumarins. The occurrence of yellow/dark-yellow color in the reaction of almost all samples (1–12, 14, 16–18, and 20) indicates the presence of coumarins, which is not surprising, considering that the leaves of almost all *Annonaceous* plants have a unique smell that is related to the presence of coumarins.

The presence of phytochemicals in the methanolic leaf extracts (1–20) is related to their antioxidant activity. The DPPH-based free radical scavenging assay was used to determine their antioxidant activities compared to that of L-ascorbic acid as a positive control. The $\%SC_{50}$ values of the samples were 5.39–74.42 µg/ml, whereby sample 1 was the most active ($\%SC_{50}$ = 5.39 µg/ml) and sample 3 was the least active ($\%SC_{50}$ = 74.42 µg/ml) among all the tested samples. The level of antioxidant activity is related to the number of hydroxyl groups, which contain lone pairs of electrons, in their structures. These lone-pair electrons of the hydroxyl groups can react with free radicals to protect against the negative health consequences of oxidative stress.

The methanolic leaf extracts showed microbiostatic activity against the test microbials. Samples 1–10 and 20 inhibited the growth of *S. aureus* at 250–500 µg/ml (sample 2 was the most active; MIC = 250 µg/ml). Samples 1–10, 12, 13, and 16–20 showed inhibitory activity against *E. coli* (MIC = 250–500 µg/ml). Samples 1–10 showed inhibitory activity against *A. baumannii* (MIC = 125 µg/ml). Samples 1–10, 13, and 16–20 showed inhibitory activity against *P. aeruginosa* (MIC = 125–250 µg/ml). Samples 1–10, 13, 17, 19, and 20 showed inhibitory activity against *K. pneumoniae* (MIC = 250–500 µg/ml). Samples 1–10

showed inhibitory activity against *E. faecalis* (MIC = 250–500 µg/ml), while sample 20 showed strong activity against *E. faecalis* (MIC = 50 µg/ml and MMC = 1,000 µg/ml). Only samples 1 and 4 showed fungistatic activity against *C. albicans* (MIC = 125 µg/ml). Based on these results, it seems feasible to conclude that the antimicrobial activity is related to the presence of flavonoids in the methanolic leaf extracts. The specificity toward the microbes depends on the structures of the compounds contained in the methanolic leaf extracts. Among these samples, sample 20 was particularly interesting as it showed bactericidal activity against *E. faecalis* (MIC = 50 µg/ml and MMC = 1,000 µg/ml), as were samples 1 and 4, which showed fungistatic activity against *C. albicans* (MIC = 125 µg/ml). This is the first time to report on the biological activities of all samples, especially sample 20.

CONCLUSION

Flavonoids were found in the methanolic leaf extracts of all *Annonaceous* plants in this study. Alkaloids, acetogenins, coumarins, and saponins are distributed in some kinds of *Annonaceous* plants. The specific activities of the methanolic leaf extracts are related to the types and amounts of their components. In this study, the antioxidant and antimicrobial activities of the methanolic leaf extracts of 20 *Annonaceous* plants were determined. All samples contained flavonoids, and all showed antioxidant activity in the DPPH assay. The potency of each sample depends on the substituent groups in the structure of the flavonoids, such as hydroxyl groups, which contain lone pairs of electrons and are able to react with free radicals. Based on the antimicrobial activity results of the test samples, the antimicrobial activity can be expected to be related to the presence of flavonoids in the methanolic leaf extracts. The specificity of the antimicrobial activity of the methanolic leaf extracts toward the microbes depends on the structures of the compounds they contain. Among these samples, extract 20 was particularly interesting as it showed bactericidal activity against *E. faecalis* (MIC = 50 µg/ml and MMC = 1,000 µg/ml), as were extracts 1 and 4, which showed fungistatic activity against *C. albicans* (MIC = 125 µg/ml). These three samples can thus be considered promising potential candidates for further investigations in order to obtain more scientific data and harvest the health benefits of the active compounds. This research was run on the theme of the discovery of the polypharmacophore from natural products. The main activity was antimicrobial activity. The microbial infection related to NO and oxygen free radicals leads to degenerative symptoms. The sample which showed antimicrobial activities with antioxidant activity will be a good candidate for medicine with synergistic activity. Among 20 species of *Annonaceous* plants from Nakhon Si Thammarat, samples 1 and 2 were those candidates. On the other hand, this is the first report on the biological activities of sample 20. However, all results in this research will be the guide for drug discovery of *Annonaceous* plants.

Patents

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AUTHORS' CONTRIBUTIONS

All authors conceived and designed the study. BW conducted the experiments, analyzed the data, and wrote the paper. All authors contributed to manuscript revisions. All authors have approved the final version of the manuscript and agree to be held accountable for the content therein.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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SUPPLEMENTARY MATERIALS

Antibacterial and antioxidant activities of methanolic leaf extracts of some *Annonaceous* plants found in Nakhon Si Thammarat, Thailand

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Supplementary Table S1. Phytochemical screening of methanolic leaf extracts of some *Annonaceous* plants in Nakhon Si Thammarat, Thailand (Sample1–20) (Auwal *et al.*, 2014; El Hazzam *et al.*, 2020; Heera *et al.*, 2012; Hossain *et al.*, 2013; Raal *et al.*, 2020; Stefanachi *et al.*, 2018).

Sample No.	Plant name	Dragendorff test	Liebermann–Burchard test	Ammonium hydroxide TS	Raymond's reagent	Keddé's reagent	Forth test	1% $FeCl_3$	Sat .lead subacetate	6 M NaOH
1	*Miliusa lineata*	-	Magenta	Red-orange solution with yellow precipitate (+)	-	+	++	Turquoise precipitate	Precipitate (+)	Dark yellow
2	*Polyalthia suberosa*	-	Blue green and magenta	Red-orange solution with yellow precipitate (++)	++	-	++	Turquoise precipitate	Precipitate (++)	Dark yellow
3	*Anaxagorea javanica*	+	Blue green and magenta	Yellow solution with yellow precipitate (+++)	-	-	+	Turquoise precipitate	Precipitate (+++)	Dark yellow
4	*Desmos chinensis*	+	Magenta	Red-orange solution with yellow precipitate (+)	-	-	-	Black precipitate	Precipitate (+++)	Dark yellow
5	*Uvaria rufa*	-	Magenta	Red-orange solution with yellow precipitate (++)	+++	+++	+	Turquoise precipitate	Precipitate (+++)	Dark yellow
6	*Winitia cauliflora*	-	Magenta	Yellow solution with yellow precipitate (+++)	-	-	+++	Turquoise precipitate	Precipitate (++)	Dark yellow
7	*Uvaria curtisii*	-	Magenta	Yellow solution with yellow precipitate (+)	++	+	++	Turquoise precipitate	Precipitate (++)	Dark yellow
8	*Frisodielsia desmoids*	-	Magenta	Red-orange solution with yellow precipitate (+)	+	+	++	Turquoise precipitate	Precipitate (++)	Dark yellow
9	*Platymitra macrocarpa*	++	Blue green and magenta	Red-orange solution with yellow precipitate (+)	++	+++	-	Turquoise solution	Precipitate (++)	Dark yellow

Sample No.	Plant name	Dragendorff test	Liebermann–Burchard test	Ammonium hydroxide TS	Raymond's reagent	Keddé's reagent	Forth test	1% $FeCl_3$	Sat .lead subacetate	6 M NaOH
10	*Rauwenhoffia siamensis*	-	Blue green and magenta	Yellow solution with yellow precipitate (+++)	-	-	-	Turquoise precipitate	Precipitate (++)	Dark yellow
11	*Dasymaschalon blumei*	++	Magenta and black	Red-orange solution (-)	+	++	+++	Turquoise precipitate	Precipitate (+)	Yellow
12	*Desmos cochinchinensis*	-	Blue green and magenta	Yellow solution with yellow precipitate (+++)	+	+	-	Turquoise precipitate	Precipitate (++)	Yellow
13	*Goniothalamus tapis*	-	Blue green and magenta	Yellow solution with yellow precipitate (++)	+	+	++	Turquoise precipitate	Precipitate (++)	-
14	*Alphonsea elliptica*	++	Blue green and magenta	Yellow solution with yellow precipitate (+++)	+	+	++	Turquoise precipitate	Precipitate (+)	Yellow
15	*Uvaria grandiflora*	+	Blue green and magenta	Yellow solution with yellow precipitate (++)	+	-	+	Turquoise precipitate	Precipitate (+)	-
16	*Artabotrys hexapetalus*	-	Blue green and magenta	Yellow solution with yellow precipitate (+)	+	+	-	Black precipitate	Precipitate (+++)	Dark yellow
17	*Cananga odorata*	-	Blue green and magenta	Yellow solution with yellow precipitate (++)	+	+	+	Turquoise precipitate	Precipitate (++)	Yellow
18	*Annona squamosa*	-	Blue green and magenta	Yellow solution with yellow precipitate (++)	+	+	-	Turquoise precipitate	Precipitate (+)	Yellow
19	*Sageraea elliptica*	-	Blue green and magenta	Yellow solution with yellow precipitate (++)	-	-	+	Turquoise precipitate	Precipitate (+)	-
20	*Mitrephora chulabhorniana*	-	Blue green and magenta	Yellow solution with yellow precipitate (+)	-	-	-	Turquoise precipitate	Precipitate (++)	Dark yellow

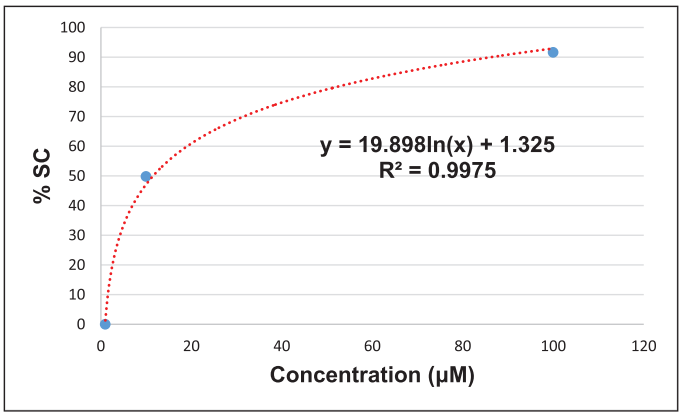

Figure S1. DPPH radical scavenging activity of **Extract 2,** SC_{50} = 11.55 µM (Akaike *et al*., 2001; Braca *et al*., 2001).

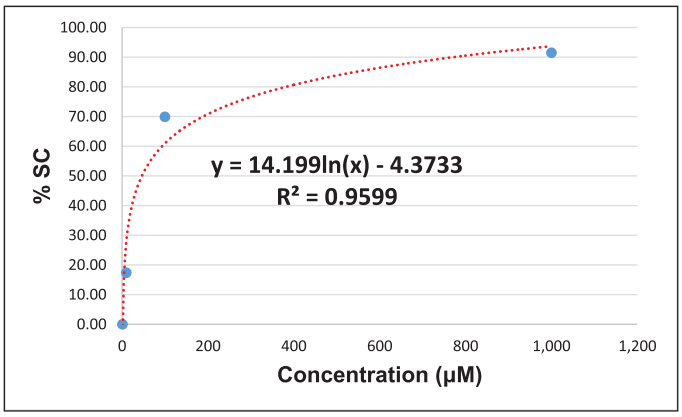

Figure S3. DPPH radical scavenging activity of **Extract 6,** SC_{50} = 46.04 µM.

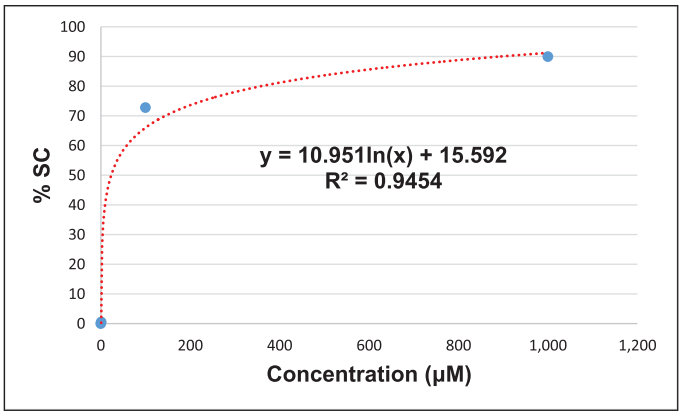

Figure S2. DPPH radical scavenging activity of **Extract 4,** SC_{50} = 23.14 µM.

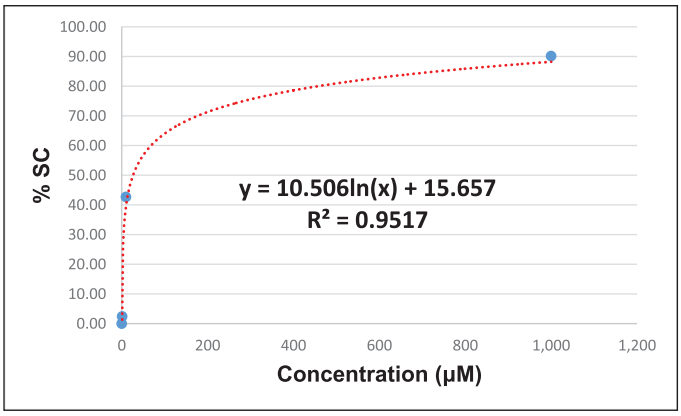

Figure S4. DPPH radical scavenging activity of **Extract 8,** SC_{50} = 26.28 µM.