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Phytochemical Screening and Antibacterial Properties of Methanol, Hexane and Water Extracts of *Clinacanthus nutans*

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Clinacanthus nutans (*C. nutans*) is classified under the Acanthaceae plant family found in Southeast Asia, and has been used as ethnomedicine to treat bacterial infections. In the literature to date, there is a lack of information on the phytochemical constituents in *C. nutans* leaf extracts and their pharmacological actions, especially their antibacterial properties. In this study, we performed a phytochemical analysis on the methanol, hexane and water extracts of *C. nutans*. Different chemical tests were used to screen for the presence of alkaloids, flavonoids, saponins, terpenoids and tannins in the extracts. The preliminary phytochemical screening showed that terpenoids were found in all extracts, while flavonoids and tannins were only found in the water extract, and saponins were only found in the methanol extract. Thin layer chromatography (TLC) analyses confirmed that alkaloids were absent, but various flavonoids were present in all the extracts. In addition, all the extracts were able to inhibit the growth of *S. aureus* and *B. subtilis*, with zone of inhibition diameters of ~7-10 mm. However, no inhibition was observed for all extracts against *E. coli*. The MIC values for the water and methanol extracts against *S. aureus* were similar (12.50 mg/mL). Interestingly, the water extract was twice as effective (25.00 mg/mL) as the methanol extract (50.00 mg/mL) in inhibiting *B. subtilis*. The minimum inhibitory concentration (MIC) values for the hexane extract could not be determined as the extract precipitated after the addition of broth, which resulted in the formation of a cloudy solution. The antibacterial activity exhibited by all *C. nutans* extracts against *S. aureus* and *B. subtilis* suggests their antibacterial potential towards Gram-positive bacteria.

Keywords: *Clinacanthus nutans*; flavonoids; tannins; antibacterial property

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C. nutans has been widely used as a traditional medicine for decades due to its beneficial pharmacological actions. The leaves of *C. nutans* are extracted using alcohol and utilized as a topical application to treat insect and snake bites, skin rashes, varicella-zoster virus (VZV) and herpes simplex virus (HSV) infections in Thailand [1]. The leaves can be taken directly by mouth or as a health drink when mixed with other beverages such as sugarcane, apple juice or green tea [2].

In Malaysia, a preliminary screening of the active phytochemicals in ten medicinal plants including *C. nutans* was performed in Pulau Bruit, Sarawak [3]. 95 % undenatured ethanol was used as the solvent for the plant extractions and results showed that *C. nutans* contained a variety of phytochemicals (alkaloids, flavonoids, saponins and tannins). Another study on the phytochemical analysis of the stems and leaves of *C. nutans* was also conducted using qualitative and quantitative methods [4]. The researchers used 70 % acetone to prepare the plant extracts and subjected them to phytochemical screening. Various phytochemicals such as flavonoids, alkaloids, saponins, glycosides, steroids, phenols, tannins and terpenoids were found in the extracts [4]. However, other types of extracts such as water, hexane and methanol extracts were not examined in this study [3].

Natural products have become an important source of new treatments for bacterial infections. The acetone extracts of *C. nutans* stems and leaves were prepared to investigate their MIC values using the broth macrodilution method and minimum microbicidal concentration (MMC) test [5]. With 70 % acetone as the extraction solvent, the antibacterial activity of the *C. nutans* leaf extract against *B. subtilis* was 12.50 mg/mL, while that of the stem extract against *E. coli* was 25.00 mg/mL [5]. An investigation on the antibacterial property of *C. nutans* leaf and stem extracts using polar (methanol and dichloromethane) and non-polar solvents (hexane and diethyl ether) was carried out against twelve Gram-positive and Gram-negative bacteria [6]. In general, the higher the concentration of the extracts, the more effective they were against all the tested bacteria species. In addition, the *C. nutans* leaf extracts with non-polar solvents exhibited more effective inhibitory action compared to the leaf extracts with polar solvents [6]. However, the phytochemicals responsible for the

inhibitory activity were not identified in this study. Although *C. nutans* is commonly found in Southeast Asia, only certain types of phytochemicals have been studied by local researchers [7]. Despite there being some studies on the pharmacological actions of *C. nutans* extracts, the research published to date is limited. Therefore, in this study, the phytochemical constituents and antibacterial activity of the methanol, water and hexane extracts of *C. nutans* were determined and compared.

EXPERIMENTAL

C. nutans Leaf Extracts

The methanol, n-hexane and water extracts of *C. nutans* leaves were obtained as described in our previous study [8]. 100 g of powdered *C. nutans* leaves was stirred in hexane, methanol or distilled water for 48 h at room temperature. Each sample was filtered and concentrated using a rotary evaporator at 60 °C. The dried extracts were stored at -20 °C and diluted to 0.1 g/mL with methanol for further analysis.

Test for Alkaloids

100 µL of the methanol, hexane and water extracts of *C. nutans* were transferred into three tubes. 50 µL of 1 % hydrochloric acid (HCl) was added to each tube, followed by 50 µL of Dragendorff's reagent. The presence of alkaloids was indicated by the formation of an orange-red precipitate [9].

Test for Flavonoids

500 µL of the methanol, hexane and water extracts of *C. nutans* were transferred into three tubes. A magnesium strip was added to each tube, followed by 50 µL of concentrated HCl. The mixtures were placed in a warm water bath for 5 minutes. A positive result was indicated when the mixture turned red-pink [10].

Test for Saponins

Three tubes were filled with 1 mL of each extract and 5 mL of distilled water and then shaken vigorously for 15 minutes. Persistent frothing demonstrated the presence of saponins [11].

Test for Tannins

50 µL of each extract was mixed with 20 µL of distilled water and warmed for 5 minutes in a water bath. 20 µL of 1 % ferric chloride was added to each sample. Tannins were deemed present if the mixture turned brownish-green or blue-black [12].

Test for Terpenoids

2.5 mL of each extract was mixed with 1 mL of chloroform, while 1.5 mL of concentrated sulfuric acid was added dropwise. The formation of a reddish-brown colour at the interface indicated the presence of terpenoids [13].

TLC Analysis for Alkaloids and Flavonoids

TLC analysis of the plant extracts was performed using the method described by Biradar and Tiwari et al. [14,15]. The standards used for alkaloids and flavonoids were caffeine and quercetin, respectively. Dragendorff's reagent and 5 % ethanolic aluminium chloride were used as sprays for alkaloids and flavonoids, respectively. The plate was visualized under UV light at 365 nm [14,15].

Antibacterial Screening of *C. nutans* Extracts Disc Diffusion Method

Antibacterial screening was performed based on the disc diffusion method with a little modification [16,17]. 100 µL of *E. coli*, *B. subtilis* and *S. aureus* were each pipetted from 24 h bacterial suspensions and spread over the agar plates. Using sterilized forceps, sterile filter paper discs filled with 10 µL of methanol (negative control) and 10 µL of plant extract (with a final concentration of 1 mg/mL) were transferred onto the agar plates. At the same time, discs impregnated with 10 µg tetracycline, ampicillin or gentamycin (Oxoid, UK) were used as the positive controls. The agar plates were incubated at 37 °C for 24 h. Lastly, the diameters of the zones of inhibition around the discs were measured using a Vernier calliper. Measurements were performed in triplicate, and average readings were obtained.

Determination of MIC

MIC assays were performed according to the procedure described by Saeloh et al. with minor modifications [18]. The Mueller Hinton broth (MHB, Oxoid, UK) cultures of the bacteria were adjusted so that the turbidity was equivalent to 0.5 McFarland standard (~1 x 10^8 CFU/mL). The adjusted suspension was diluted 100 times using MHB to prepare a final bacterial concentration of 5 x 10^5 CFU/mL [19]. 100 µL of each extract (3.125, 6.25, 12.5, 25 and 50 mg/mL) was added to a 96-well plate which contained 100 µL of the diluted bacterial suspension. The plate was incubated for 24 h at 37 °C.

Table 1. Phytochemicals in the methanol, hexane and water extracts of *C. nutans.*

Phytochemicals	Control (methanol)	Methanol extract	Hexane extract	Water extract
Alkaloids	-	++	++	+
Flavonoids	-	-	-	+
Saponins	-	+	-	-
Tannins	-	-	-	+
Terpenoids	-	++	++	++

Notes: + weak precipitate/weak positive, ++ strong precipitate/strong positive, - no precipitate/negative

RESULTS AND DISCUSSION

Phytochemical Screening of *C. nutans*

Five phytochemical tests were used to identify the constituents of the methanol, hexane and water extracts of *C. nutans* leaves. Dragendorff's test, Shinoda's test, the foam test, the Salkowski test and Braymer's test were used to detect the presence of alkaloids, flavonoids, saponins, terpenoids and tannins, respectively. Alkaloids were present, as indicated by orange-red precipitates, in all extracts. In addition, terpenoids were also present in all extracts, where the formation of a reddish-brown ring indicated the presence of phytosterols, which are a subclass of terpenoids [13]. Flavonoids and tannins were found only in the water extract, whereas saponins were present only in the methanol extract (Table 1). In contrast to our findings, some studies found that the methanolic extract of *C. nutans* leaves contained flavonoids [20,21], but alkaloids [20-22] and saponins [22] were not detected. Different extraction methods and solvent concentrations may affect the phytochemical constituents of the plant extract [23].

The absence of phenolic compounds such as flavonoids and tannins in the hexane extract of *C. nutans* leaves was in line with a previous study, which showed that the hexane extract contained the lowest mean total phenolic content [8]. Tannins were identified in the study using the cold extraction method instead of maceration [24]. In addition, an investigation demonstrated that the total tannin content and total flavonoid content of the n-hexane extracts were the lowest and second lowest, respectively, compared to the ethanol, ethyl acetate and dichloromethane extracts [25].

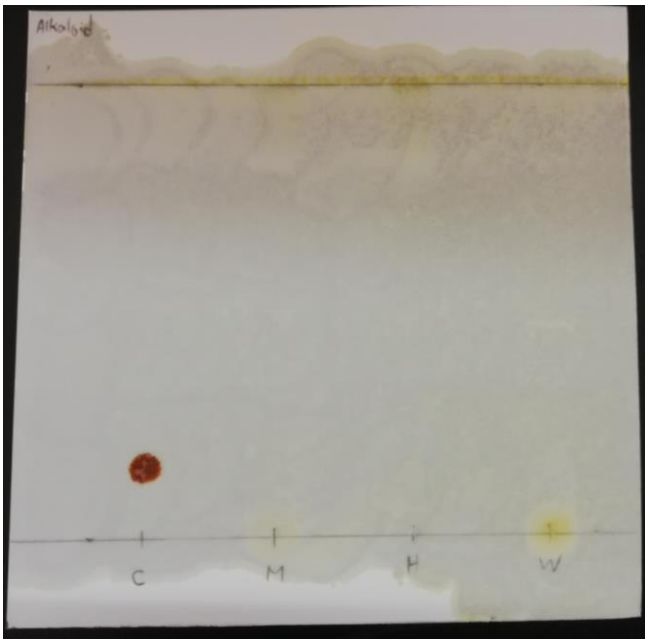

Figure 1. TLC of *C. nutans* extracts for the analysis of alkaloids after applying Dragendorff's reagent. C: Caffeine (standard); M: Methanol extract; H: Hexane extract; W: Water extract.

The water extract of *C. nutans* displayed positive results for alkaloids, flavonoids and tannins, but saponins were not detected (Table 1). These findings were in line with the results of a study in Indonesia [26]. However, according to another study, alkaloids and tannins were not found in water extracts, which may be attributed to the use of a different extraction technique (Soxhlet extraction) [27]. There is a high possibility of thermal degradation of phytochemicals due to the high temperatures used in Soxhlet extractions [28] which resulted in a decrease in the total alkaloid content of *Camellia sinensis L.* extracts, when compared with extraction at lower temperatures [29].

TLC Analyses

Figure 1 shows the TLC for the analysis of alkaloids in the methanol, hexane and water extracts of *C. nutans* leaves after applying the visualizing agent (Dragendorff's reagent). Only one spot was seen on the caffeine standard (Figure 1) and the absence of spots in all three extracts indicated that they did not contain alkaloids. This result contrasted with the result from the preliminary phytochemical screening, in which the Dragendorff's tests were positive for all the extracts. This may be due to the preliminary phytochemical screening sometimes giving false positive or negative results [30]. Therefore, more specific and sensitive methods may be required to further verify the results.

There has been little research on flavonoids in *C. nutans* extracts by TLC analysis. However, one study demonstrated that the types of flavonoids in *Ficus sycomorus*, *Carissa bispinosa* and *Grewia bicolar* fruit extracts could be determined by TLC analyses based on the different colours of the bands [31]. The TLC plate for the analysis of flavonoids in the methanol, hexane and water extracts observed under UV light at 365 nm after applying 5 % ethanolic aluminium chloride is shown in Figure 2.

Quercetin is a flavonol, and the green bands in Figure 2 indicate the presence of flavonols and flavones [32,33]. The orange-brown bands may indicate the presence of flavonol glycosides in the *C. nutans* water and methanol extracts [31,34]. Flavone glycoside biflavonols and unusually substituted flavones may be present in the hexane extract, as indicated by the light-yellow band [34] (Figure 2). These findings are in line with a study in which a light-yellow fluorescence was observed for flavone C-glycosides with a luteolin backbone [35]. The blue fluorescence observed in the water extract indicates the presence of 7,8-dihydroxy-flavanones, 5-deoxyisoflavones or anthocyanidin 3,5-diglycosides [34,36]. From this evaluation, flavonoids were present in all three extracts.

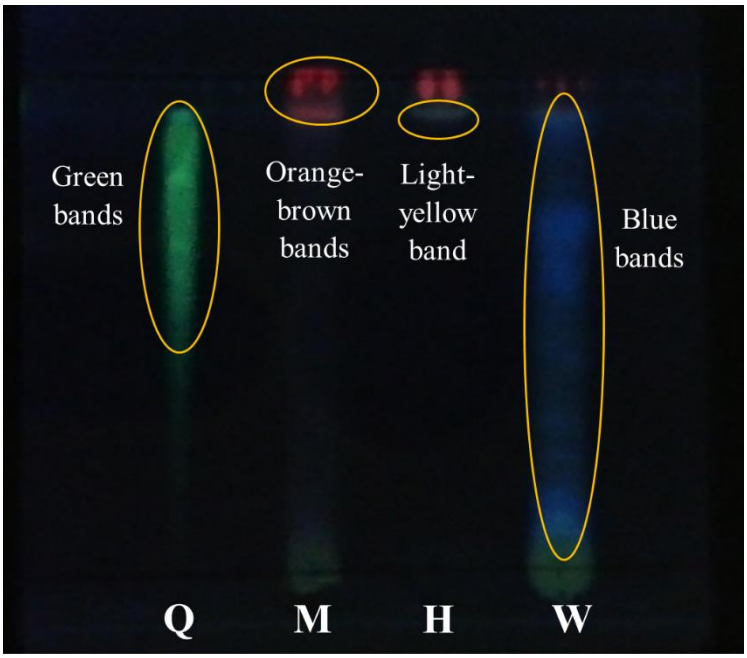

Figure 2. TLC plate under UV light (365 nm) of *C. nutans* extracts for the analysis of flavonoids. Bands with different colours are indicated using yellow circles. Q: Quercetin (standard); M: Methanol extract; H: Hexane extract; W: Water extract.

Table 2. The antibacterial activities of different extracts of *C. nutans* leaves using the disc diffusion method.

Sample	Diameter of zone of inhibition (mm)		
	Gram-positive bacteria		Gram-negative bacteria
	S. aureus	***B. subtilis***	***E. coli***
Negative control			
Methanol	-	-	-
Positive control			
Ampicillin	13.50 ± 1.84	8.23 ± 0.65	17.90 ± 0.95
Tetracycline	27.57 ± 0.81	21.47 ± 1.25	25.73 ± 0.74
Gentamycin	22.07 ± 0.35	18.00 ± 0.46	20.03 ± 0.81
Methanol extract	9.50 ± 2.97	8.10 ± 0.71	-
Hexane extract	9.17 ± 1.42	8.00 ± 1.84	-
Water extract	9.95 ± 4.03	6.90 ± 0.28	-

Note: The diameter of the zone of inhibition was expressed as the mean ± standard deviation with n = 3. - : no inhibition observed.

Antibacterial Screening

Methanol was used as the negative control. 10 µg ampicillin, tetracycline and gentamycin were the three antibiotics which acted as the positive controls. The diameters of the inhibition zones for the antibiotics against *S. aureus*, *B. subtilis* and *E. coli* ranged from ~ 8-27 mm (Table 2). All of the *C. nutans* extracts (1 mg/mL) exhibited antibacterial activity against the two Gram-positive bacteria, *S. aureus* and *B. subtilis*, with zones of inhibition of ~ 7-10 mm. However, none of the extracts inhibited *E. coli*.

Only Gram-positive bacteria were found to be inhibited by the *C. nutans* extracts. Most skin and soft tissue infections are mainly caused by Gram-positive bacteria, such as group A and group B streptococci, *S. aureus* and *Enterococcus faecalis*, compared to Gram-negative bacteria species such as *E. coli*, *Haemophilus influenzae* and Mycobacterium species [37]. This suggests that *C. nutans* extract is a potential active ingredient to be formulated as topical creams or ointments for the treatment of skin infections. The active phytochemicals which may exhibit antibacterial properties are saponins [38], flavonoids [39] and tannins [40].

Determination of MIC

Table 3 shows the MIC values of the three different extracts of *C. nutans* leaves against *S. aureus* and *B. subtilis*. The methanol and water extracts had the same MIC value against *S. aureus*, 12.50 mg/mL. The water extract was twice as effective (25.00 mg/mL) at inhibiting the growth of *B. subtilis* compared to the methanol extract (50.0 mg/mL). However, the MIC values of the hexane extract against *S. aureus* and *B. subtilis* could not be determined. The broth contained a high water content which caused the hexane extract to form a precipitate in the solution. The cloudiness of the solution increased with the increase in the concentration of the extract. In order to address this issue, we suggest the development of a microdilution technique by dissolving the extract in different solvent systems [41]. With this method, precipitate formation may be minimized.

Table 3. MIC values of the methanol, hexane and water extracts of *C. nutans* leaves against *S. aureus* and *B. subtilis.*

Extract	MIC (mg/mL)	
	S. aureus	***B. subtilis***
Methanol	12.50 ± 0.00	50.00 ± 0.00
Hexane	-	-
Water	12.50 ± 0.00	25.00 ± 0.00

A guideline for classifying the antimicrobial activity of the plant extracts using MIC values states that if the MIC value is ≤500 µL/mL, the extracts are classified as strong inhibitors, while values between 600-1,500 µL/mL are moderate inhibitors and those ≥1,600 µL/mL are weak inhibitors [42]. The MIC values of the methanol and water extracts were >1,600 µL/mL, thus, they are considered weak inhibitors of both *S. aureus* and *B. subtilis*. In order to increase the antibacterial potency, a purified compound needs to be identified, instead of the total herbal extract [43].

CONCLUSION

In this study, methanol, hexane and water extracts of *C. nutans* were screened for phytochemicals using different chemical tests and antibacterial activity through the disc diffusion method and MIC determination. The absence of alkaloids in all extracts were confirmed by TLC analysis, which also demonstrated the presence of different types of flavonoids (flavonol glycosides, flavone glycosides, 7,8-dihydroxy-flavanones) in all three extracts. Saponins and tannins were only found in the methanol and water extracts, whereas, terpenoids were found in all extracts. On the other hand, all the *C. nutans* extracts displayed inhibition of *S. aureus* and *B. subtilis* (~7-10 mm). However, none of the extracts inhibited *E. coli*; this may be due to the morphological and structural differences in the cell walls of Gram-positive and Gram-negative bacteria. The methanol and water extracts had the same MIC value of 12.50 mg/mL against *S. aureus*. The MIC value of the water extract (25.00 mg/mL) was half the value of the methanol extract (50.00 mg/mL) for the inhibition of *B. subtilis*. These results reveal the promising antibacterial potential of different *C. nutans* extracts against Gram-positive bacteria.

However, the MIC value of the hexane extract could not be determined due to the cloudy mixture formed after addition of the bacterial suspension. Therefore, a fluorescence-based dye is recommended to be used as an indicator to quantify the survival of bacterial cells. An example is resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), a blue dye that will be reduced to pink by metabolically active bacteria cells [44].

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