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Antioxidant, antimicrobial and total phenolic contents of *Calophyllum symingtonianum*

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PEER REVIEW

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Comments

This is a valuable research work in which author has successfully demonstrated the antioxidant and antimicrobial characteristics of the stem bark of the *C. symingtonianum in vitro*.

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ABSTRACT

Objective: To determine the total phenolic content, antioxidant and antimicrobial activities of the extracts from *Calophyllum symingtonianum*.

Methods: The extracts were tested for their antioxidant activity by the DPPH radical scavenging assay and the β−carotene bleaching assay, while the antimicrobial activity was determined by disc diffusion method.

Results: All the tested extracts showed antioxidant and antimicrobial properties. The extracts showed moderate antimicrobial activity against *Staphylococcus aureus* with zone of inhibition values of 10, 11 and 12 mm for *n*−hexane, dichloromethane and methanol extracts respectively at 30 μg/disc. *N*−hexane showed low antimicrobial activity against *Pseudomonas aeruginosa* (5 mm) at 30 μg/disc. The total phenolic test showed that methanol has high phenolic content (162.25 mg GAE/g of extract) compared to the other extracts.

Conclusions: The ability of the extracts to inhibit microbial growth at a concentration of 30 μg/disc indicated the its potent antimicrobial activity.

KEYWORDS

Calophyllum symingtonianum, Antioxidant, Antimicrobial, Total phenolic content

1. Introduction

Plants are magnificent chemical factories to produce different classes of metabolites such as alkaloids, flavonoids, quassinoids and betalins which have wide range of biological activity that can benefit the human[1]. Plants synthesize substances for their defences against free radicals, herbivores and microorganisms[2]. Plant extracts contain substances such as tannins, phenolics, micro elements, essential oils, peptides, unsaturated long chain aldehydes, alkaloids, steroids, flavonoids and saponin which are usually found in various parts such as roots, leaves, shoots and bark[2].

The potential antioxidant effects of plant constituents for health benefits and coronary protection as well as cancer become a major interest among researcher and industry[3]. Antioxidants are molecules capable to inhibit the oxidation by inhibiting the propagation or initiation of oxidative chain reactions[4]. It has been postulated that the antioxidant effect of plant metabolites is mainly due to phenolic compounds, such as flavonoids, phenolic acids, tannins and phenolic diterpenes[5].

The usage of synthetic antibiotics is reported to suppress the immune system thus affecting the ability of the body to fight against. In addition, pathogens have attained resistance to most of the existing antibiotics. As a result,

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it becomes highly important to explore new sources of more active and potential antibiotics. So far, plants have been proven to be the most promising source of potential antibiotics which might be safe alternative of these synthetic drugs. Several studies have been conducted on a number of plant-origin extracts and compounds have been screened for their antibacterial activities[6]. This research was conducted to study the antioxidant and antimicrobial activity of the crude extracts from the stem bark of *Calophyllum symingtonianum* (*C. symingtonianum*).

2. Materials and methods

2.1. Plant Materials

The stem barks of *C. symingtonianum* were collected from Kuantan Pahang, Malaysia. The plant (MT-26) was identified by Dr. Shamsul Khamis of University Putra Malaysia, where the specimen was deposited.

2.2. Sample extraction

The extraction procedure was conducted using the soxhlet apparatus. The sample (1.7 kg) was extracted using *n*-hexane, dichloromethane (DCM) and methanol successively. The extracts were then evaporated using the rotary evaporator to get concentrated extracts which were then stored at -4 °C for further use.

2.3. Microbial cultures

The microorganisms used were from American Type Cell Culture Collection (ATCC). A total of six bacteria were used, two Gram-negative bacteria, *Escherichia coli* ATCC35218 (*E. coli*), *Pseudomonas aeruginosa* ATCC27853 (*P. aeruginosa*), two Gram-positive bacteria, *Staphylococcus aureus* ATCC25923 (*S. aureus*), *Bacillus cereus* ATCC11778 (*B. cereus*), and two fungi, *Candida albicans* ATCC10231 (*C. albicans*), *Candida neoforman* ATCC90112 (*C. neoforman*).

2.4. DPPH free radical scavenging activity

The activity of antioxidant of the extracts and the standard was assayed based on the radical scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity using the method described[7] with the equation below:

Inhibition (%)= $((A_{DPPH}-A_{sample})/A_{DPPH})\times 100$

Where A_{DPPH}=absorbance DPPH, A_{sample}=absorbance of the sample with DPPH. Methanol with DPPH solution (0.004%) was used as blank.

About 0.1 mL of each sample and positive control in methanol to 3.9 mL of fresh DPPH solution (0.004 %) in methanol, then the mixture was incubated for 30 min, the absorbance was measured at 515 nm. The absorbance was measured using a UV/VIS spectrophotometer. All experiments were performed in triplicates.

2.5. β-carotene bleaching assay

The assay of β-carotene bleaching was conducted according to the previous method described by Velioglu *et al.*[4], in order to measure the antioxidant activity of the different extracts. β-carotene (0.2 mg) was dissolved in chloroform (1 mL) and was added to a flasks (50 mL) containing linoleic acid (20 μL) and Tween (200 μL). A total of 80% MeOH (200 μL) was used as a control or plant extract or Trolox (as standard) was added to the mixture. The concentrations of the Trolox and plant extract the same (1 mg/mL). After evaporation using rotary evaporator, 100 mL distilled water was added. The mixture was shaken to form a liposome solution. The samples were subjected to thermal autoxidation at 45 °C for 2 h. The solution absorbance was measured at 470 nm at 15 min intervals for 120 min and the rate of β-carotene bleaching was recorded.

2.6. Total phenolic content

The total phenolic content in all extracts was determined using the Folin-Ciocalteu method as described by Kassim *et al*., 2013[8]. About 5 mg of each sample was diluted in 1 mL of distilled water. A total of 1 mL of each sample was then transferred into test tubes and 5 mL of Folin-Ciocalteu were added to the samples. After 5 min, 4 mL of sodium carbonate was added to the solution and then vortex. The mixture was incubated for 2 h in the dark at room temperature. The absorbance was measured at 725 nm using a UV spectrophotometer.

2.7. Disc diffusion method

The antimicrobial susceptibility testing was carried out by disc diffusion method using the standard method previously described by Goncalves *et al*[9]. A culture of each microbial strain (adjusted to 0.5 McFarland standard), was used to lawn Muller Hinton agar plates using a Hockey stick. The plates were allowed enough time to dry before the placement of the discs impregnated with plant extracts. Each plate comprises of five discs. Streptomycin and nystatin were used as positive controls for the bacteria and fungi, respectively, while blank discs impregnated with methanol were used as negative control. The plates were then incubated at 37 °C for 24 to 48 h depending on the species of the microorganisms used in the test. After the incubation, the plates were evaluated for inhibition zone. All tests were conducted in triplicates to ensure the consistency of the results.

2.8. Minimum inhibition concentration (MIC)

The MIC was determined using the method described by Goncalves *et al*. and Gebreyohannes *et al*[9,10]. This method usually determined by using 96-well microtiter plates. Bacteria were inoculated into a liquid growth medium in

the presence of different concentrations of antimicrobial agent or plant extracts. Bacterial growth was assessed after incubation for a defined period of time (18–24 h) and the MIC value was measured.

3. Results

3.1. Total antioxidant activities

DPPH radical scavenging assay and β–carotene bleaching assay were used to determine the total antioxidant activities all extracts. Methanol extract showed higher antioxidant activity in DPPH radical scavenging assay, while hexane extract showed high antioxidant properties in β–carotene bleaching assay.

3.2. DPPH radical scavenging assay

The antioxidant activity of all extracts and positive control were assessed based on their ability to scavenge the DPPH free radicals. Figure 1 shows the IC_{50} of all extracts and positive control. IC_{50} indicated the concentration required to inhibit 50% of the DPPH free radicals. Methanolic extract showed higher radical scavenging properties with an IC_{50} of 1 mg/mL followed by DCM and hexane extracts with IC_{50} of 0.56 and 1.93 mg/mL respectively. The IC_{50} for the positive control was 0.0625 mg/mL. This indicates that only a small amount of the positive control is required to inhibit 50% of the DPPH free radicals. Figure 2 shows the antioxidant activity of all extracts and positive and negative controls.

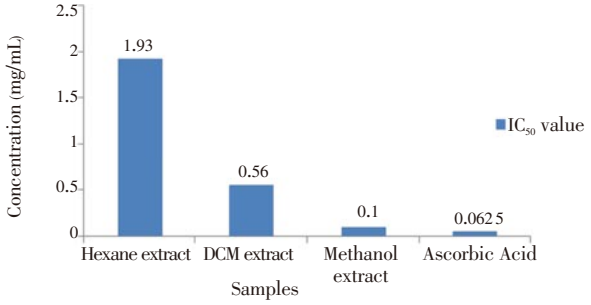

Figure 1. IC_{50} (50% inhibition concentration) values of all extracts and ascorbic acid.

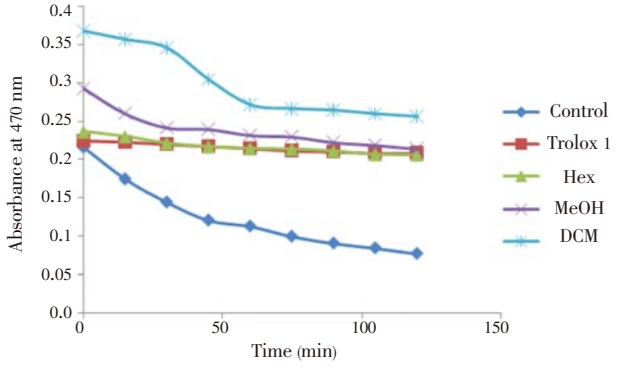

Figure 2. Antioxidant activity of all extracts and Trolox at 1 mg/mL using β–carotene bleaching assay

3.3. β–carotene bleaching assay

Beside the DPPH assay, β–carotene bleaching assay was also used to determine the antioxidant activity of extracts expressed in percentage. The result showed that *n*–hexane extract showed higher antioxidant properties (78%) (Figure 3). Figure 3 also shows that Trolox as a positive control has higher activity (88.3%) compared to all extracts.

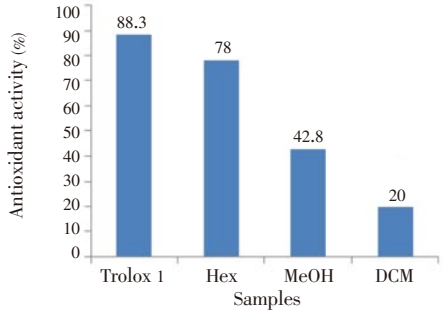

Figure 3. Total antioxidant activity of all extracts and Trolox.

3.4. Total phenolic content

Gallic acid was used as a standard compound and the total phenols were expressed as mg GAE/g of extract using the standard curve equation $Y=0.008X-0.014$, $R^2=1$ (Figure 4). Methanol extract showed higher phenolic content, 162.25 mg GAE/g extract followed by hexane and DCM extract at 69.37 and 63.29 mg GAE/g extract respectively (Table 1).

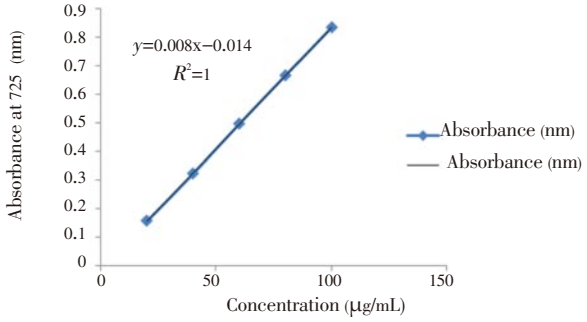

Figure 4. Gallic acid standard curve

Table 1
Total phenolic content of all extracts in mg GAE/g of the dry weight.

Samples	Phenolic content (mg GAE/g Extract)
Hexane extract	69.37
DCM extract	63.29
Methanol extract	162.25

3.5. Antimicrobial test

The *in vitro* antimicrobial properties of the crude extracts against the microbial strains were assessed by observation of inhibition zones. Methanol extract showed high antimicrobial properties compare to the other extracts. Methanol extract showed activity at 1 mg/mL against *S. aureus* with 12 mm diameter zone of inhibition. Hexane extract showed weak activity against *S. aureus* and *P. aeruginosa* at the same concentration of the methanol extract. The methanol

and hexane extracts were inactive against the rest of the microorganisms (Table 2).

Table 2
Antimicrobial activity of all extracts and positive controls.

Microorganisms	Zone of inhibition (mm)			
	n-Hexane extract	DCM extract	Methanol extract	Standard
S. aureus	10	11	12	28*
B. cereus	–	–	–	27*
E. coli	–	–	–	38*
P. aeruginosa	5	–	–	24*
C. albicans	–	–	–	17#
C. neoforman	–	–	–	16#

*Chloramphenicol; #Nystatin

3.6. Disc diffusion and minimum inhibitory concentration

The disc diffusion method was used to assess the ability of all the extracts and positive controls to inhibit the growth of the microorganisms and fungi. Methanol showed high antimicrobial activity against *S. aureus* with 12 mm diameter for the zone of inhibition. Table 3 shows the MIC value of methanol extract against *S. aureus* was 0.25 mg/mL, indicating that methanol extract was highly active as an antimicrobial agent. The MIC values for hexane extract against *S. aureus* and *P. aeruginosa* were 0.3 and 1 mg/mL respectively.

Table 3
MIC values for the extracts.

	MIC (mg/mL)			
Bacteria	n-hexane. extract	MeOH extract	DCM extract	Chloramphenicol
S. aureus	0.3	0.25	0.3	0.02
P. aeruginosa	1	–	–	–

4. Discussion

The antioxidant effect of extract on DPPH is due to their ability to donate hydrogen[11]. All extracts and positive control (ascorbic acid) were subjected for the evaluation of antioxidant activity using the DPPH radical scavenging activity test. It has been proven that the scavenging effect on the DPPH radical increases sharply with the increment amount of the samples and standards to a certain level[12]. All extracts tested positive for antioxidant.

The mechanism of β-carotene bleaching is a free radical mediated phenomenon resulting from the formation of hydroperoxides from linoleic acid[13]. Without antioxidant, β-carotene will be discoloration rapidly. Linoleic acid becomes a free radical with a hydrogen atom abstracted from its molecule. The radical formed will attack the highly unsaturated β-carotene molecules. When the β-carotene molecules lose their double bonds by oxidation, it becomes orange in colour, this process can be monitored using a spectrophotometer. The availability of antioxidants in the plant extracts can protect β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals in the system.

All the extracts showed antioxidant activity with hexane extract showing higher activity (78%) followed by methanol then DCM extract at 42.8% and 20% respectively. Phenolics are a group of phytochemicals that have been associated with most of the antioxidant activity of plants or plant products. The methanolic extract of the plant *C. symingtonianum* has shown higher phenolic content compared to hexane and DCM extracts.

The methanol and hexane extracts were inactive against the rest of the microorganisms. This could be attributed to the cell envelope including cytoplasmic membrane and cell wall components' structural differences between Gram-positive and Gram-negative bacteria[14,15]. The structural differences between the bacteria and fungi play a key role in the susceptibility. The mode of action of the antimicrobial agent also plays a vital role in the bacterial susceptibility. This is because different antimicrobial agent affects the microorganisms differently. Antimicrobial agent can affect microbes in different ways such as interfering with the protein synthesis or by affecting directly the cell wall of the microorganisms. DCM extract was inactive against all microorganisms used. The positive controls used in this test were streptomycin and nystatin for bacteria and fungi respectively.

The results indicated that the stem bark extracts of *C. symingtonianum* possess antioxidant and antimicrobial properties and could be used to scavenge or inhibit free radicals. The methanol and hexane extracts of *C. symingtonianum* has shown higher antimicrobial activity against *S. aureus*. This indicates that the extracts have antimicrobial compounds which can be developed into new antimicrobial drugs. Higher phenolic content were also recorded from the methanolic content. Since major attention has been paid to natural sources of antioxidant and antibacterial agents, the results from this study suggest a possible use of *C. symingtonianum* as a source of natural antioxidant and antimicrobial agents.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The antioxidant effect of plant constituents for health benefits and coronary protection as well as cancer has become a major interest among researcher and industry. Moreover, plants have been proven to be the most promising source of potential antibiotics which might be safe alternative of synthetic drugs. Hence, this research was done to evaluate the antioxidant and antimicrobial potential of the crude extracts of the stem bark of *C. symingtonianum*.

Research frontiers

The present research work depicts antioxidant and antimicrobial potential of the crude extracts of the stem bark of *C. symingtonianum* and was assessed by estimating different biochemical paradigms and *in-vitro* antioxidant and antimicrobial parameters.

Related reports

DPPH and β-carotene bleaching assays are well established methods to determine antioxidant potential of plant extracts. It is well known that the antioxidant effect of extract on DPPH is due to their ability to donate hydrogen and the mechanism of β-carotene bleaching is a free radical mediated phenomenon resulting from the formation of hydroperoxides from linoleic acid. Many studies have evidenced about the superiority of these methods to explore antioxidant potential of plant extracts as well as individual constituents *in vitro*. The antimicrobial test was conducted using the disc diffusion method and minimum inhibitory concentration of each extract was also determined which is considered essential about the authenticity of antimicrobial agents.

Innovations and breakthroughs

In the present work, author has explored the antioxidant and antimicrobial potential of the stem bark of *C. symingtonianum in vitro*.

Applications

In future studies, antioxidant and antimicrobial potential of this plant could further be evaluated in order to isolate individual constituents responsible for antioxidant and antimicrobial effects. This scientific study support and suggest the use of this plant as an adjuvant along with commonly used antimicrobial as well as anticancer agents.

Peer review

This is a valuable research work in which author has successfully demonstrated the antioxidant and antimicrobial characteristics of the stem bark of the *C. symingtonianum in vitro*. The antioxidant activity was assessed based on DPPH and β-carotene bleaching assays and antimicrobial activity was evaluated by following disc diffusion method and MIC was determined by using 96-well microtiter plates.

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