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Original Research Article

Antibacterial potential of extracts of various parts of *Catunaregam tomentosa* (Blume ex DC) Tirveng and their effects on bacterial granularity and membrane integrity

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Abstract

Purpose: *To investigate the antibacterial activity of extracts from Catunaregam tomentosa on Bacillus subtilis and Staphylococcus aureus, and the bacterial responses to the extracts.*

Methods: *The antibacterial activity of fruit, leaf and stem bark extracts were evaluated against B. subtilis (ATCC6633) and S. aureus (ATCC25923). Using a disc diffusion method, extracts at concentrations ranging from 50 – 1,000 µg/disc were tested. The minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of the extracts against the test bacteria were determined. Fluorescent activated cell sorting (FACS) was used to assess the responses of both types of bacteria to the extracts.*

Results: *The fruit and leaf extracts at 1,000 µg/disc showed optimum efficacy against B. subtilis and S. aureus with MIC of 1,000 µg/mL against both B. subtilis and S. aureus, for the fruit and the leaf extracts. With increasing doses of fruit and leaf extracts at 6 h of incubation, FACS profiles revealed that cell death for B. subtilis increased. The fruit and leaf extracts of C. tomentosa also exhibited antibacterial activity against S. aureus in a dose- and time-dependent manner. The bacteria initially lost their granularity, then lost membrane integrity, and consequently died.*

Conclusion: *The fruit and leaf extracts of C. tomentosa exhibit significant antibacterial potential against Gram-positive bacteria by damaging bacterial granularity and membrane integrity.*

Keywords: *Catunaregam tomentosa, Flow cytometry, Programmed cell death, Response pattern, Bacterial granularity, Membrane integrity*

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INTRODUCTION

In recent years multidrug-resistant (MDR) pathogenic bacteria have been increasingly reported, largely due to a history of indiscriminate use of various antibiotics. This process selects for the resistant strains, killing off the competing non-resistant bacterial population. MDR strains

are therefore common in connection with infections, such as gastritis, urinary tract infections, encephalitis, and food poisoning [1,2]. In addition, in 2014, the World Health Organization reported that MDR strains are now a major threat to public health, as no antibiotic has efficacy against them.

Thus, it is necessary to search for novel alternatives, using plant and animal natural products as potential sources of antibacterial drugs. Traditional medicine tends to rely on medicinal herbal plants, and such medicinal plants may provide novel phytochemicals to use as bactericides [3]. The use of medicinal herbal plants has been promoted as effective against infectious diseases, but with fewer side effects than synthetic drugs.

Many plant families are of current interest in medicinal research. Interestingly, various parts of the plants in the genus Rubiaceae have been reported to have significant antibacterial efficacies [4,5]. For instance, the roots of *Catunaregam tomentosa* reportedly have antibacterial properties against some Gram positive bacteria [6]. In traditional medicine, the roots of *C. tomentosa* have been used by healers for stomatitis and ulcer therapies. However, in Thai traditional medicine, while the roots of this plant are designated for treating infectious symptoms and *in vivo* for antibacterial effects, the aerial parts of the plant, such as fruit, leaf, and stem bark, have not been measured for their potential bioactive activity.

In the present study, we tested the activities of *C. tomentosa* fruit, leaf, and stem bark extracts against *Bacillus subtilis* and *Staphylococcus aureus,* and assessed the bacterial population responses to these extracts.

EXPERIMENTAL

Plant materials and extract preparation

Fresh plant tissue samples (fruit, leaf, and stem bark) were collected from 10-year old *C. tomentosa* from the Chaiya District, Suratthani Province, Thailand. The samples were washed separately under running tap water and dried. Each dried sample (125 g) was soaked in 99% methanol for 5 days, and the suspension was filtered with two cotton layers. The methanolic filtrate was concentrated in a rotary evaporator at 45 ºC until a sticky mass was obtained. The sticky mass was weighed and then stored at 4 ºC until further use. These constituted the three concentrated extracts, representing fruit, leaf, and stem bark.

Test microorganisms

The bacterial strains tested for antimicrobial activity by *C. tomentosa* extracts were *B. subtilis* (ATCC6633), and *S. aureus* (ATCC25923). The bacterial strains were obtained by and maintained at the Scientific Laboratory and Equipment Center, Prince of Songkla University, Surat Thani campus, Thailand.

The bacterial strains were cultured on nutrient agar (NA) plates and incubated for 18 to 24 h at 37 ºC. A single colony was then cultured in 50 mL nutrient broth (NB) for 4 h at 37 ºC. The turbidity of the bacterial culture required for the tests was adapted to 0.5 McFarland standard, (1.0×10^7 colony forming units/mL) and was measured with a spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA).

Disc diffusion assay

Antibacterial activities of the extracts were estimated by the disc diffusion method on trypticase soy agar. Sterile cotton swabs were dipped into the bacterial strain suspension (at 1×10^7 CFU/mL) and used to uniformly inoculate the whole surface of an agar plate. Filter paper discs (6 mm diameter) were used to absorb 25 µL of an extract in aqueous solution, at concentrations that gave 50, 100, 250, 500, or 1,000 µg/disc of the extract. The discs were air dried and placed on the inoculated agar. The agar plates were then incubated for 24 h at 37 ºC. The standard antibiotic disc (positive control) had oxytetracycline (OTC) at 30 µg/disc, and the negative control discs contained 10% dimethyl sulfoxide (DMSO).

Determination of minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC)

MIC was determined for each type of plant extract that gave clearly positive results in the disc diffusion test**.** The determination used two-fold serial dilutions, with extract concentrations of 1,000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, and 1.95 µg/mL. One mL of extract solution was added to the first tube containing 1 mL of sterile trypticase soy broth with 1×10^7 CFU/mL bacteria, resulting in a final concentration of extract of 1,000 µg/mL. Then, 1 mL of this solution was transferred to the next tube containing 1 mL of 1×10^7 CFU/mL bacteria, for a two-fold dilution.

These steps were repeated nine times to create the dilution series. DMSO served as the negative control, and the positive control was

oxytetracycline at 30 µg/mL. The culture was incubated at 37 ºC for 24 h. The MIC was determined as the final dilution of extract that showed growth inhibition of the microorganisms. The MBC was determined after the MIC determination. The cultures in the tubes that showed no turbidity were each spread on an NA plate. The plates were incubated overnight at 37 ºC, and the lowest concentration without bacterial growth was recorded as the MBC.

Fluorescent activated cell sorting (FACS) analysis

Bacterial strains were grown to the mid-logarithmic phase (1×10^7 CFU/mL), and then the cultures were harvested and 1 mL of each bacterial strain was placed in a microcentrifuge tube. Each sample was centrifuged at 8,000 rpm for 10 min, and then the extracts were added at concentrations of 0.5 × MIC, 1 × MIC, and 2 × MIC. While exposed to a range of extract concentrations, the bacteria were incubated at 37 ºC for 3 h and 6 h. After incubation, the samples were centrifuged at 8,000 rpm for 10 min, and then washed twice with 950 µL phosphate-buffered saline. Each sample was mixed with propidium iodide (PI; 30 µg/mL) and incubated for 15 min at room temperature in darkness, before testing with flow cytometry.

Flow cytometry procedures and interpretations of the FACS profiles followed previously published methods [7]. Briefly, when a density plot of the cell population was made in the PI and side scatter (SSC) quadrants, the treated cells were labeled according to their quadrant: lower left represented viable cells (PI-, SSC-), lower right represented membrane-damaged cells (PI+, SSC-), upper left represented injured cells (PI-, SSC+), and upper right represented dead cells (PI+, SSC+). The transition from healthy viable bacteria to those killed by a treatment was assessed from the FACS data. The percentage of dead bacteria were also counted and compared between alternative treatments.

Statistical analysis

The data are presented as mean ± SD and were analyzed by analysis of variance (ANOVA). Comparisons of the means between treatments were performed using the Duncan's Multiple Range test. $P < 0.05$ was considered statistically significant. The data obtained from antibacterial studies were analyzed using SPSS statistical software, version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Antibacterial activity

The antimicrobial activities of *C. tomentosa* fruit, leaf, and stem bark extracts were studied with two types of Gram positive bacteria. The extract exhibited antibacterial properties as shown in Table 1. The zones of inhibition by the leaf extract against *B. subtilis* and *S. aureus* were from 8.0 mm–12.67 mm, and the 1,000 µg/disc dose was the most effective. The maximal inhibition zones were observed with the fruit extract of *C. tomentosa* at 1,000 µg/disc, these being 12.23 mm with *B. subtilis* and 12.17 mm with *S. aureus*. Thus, both types of bacteria were sensitive to the fruit and the leaf extracts of *C. tomentosa*. However, the stem bark extract did not exhibit antibacterial activity against the tested bacteria. Therefore, only the fruit and leaf extracts were used in subsequent experiments.

Table 1: *In vitro* antibacterial activity of the plant extract

Dose of extract (µg/disc)	Diameter of zone of inhibition (mm)					
	Bacillus subtilis			***Staphylococcus aureus***		
	Fruit extract	**Leaf extract**	**Stem bark extract**	**Fruit extract**	**Leaf extract**	**Stem bark extract**
OTC						
30	22.67±0.57^{a}	23.33±1.53^{a}	22.67±1.53	35.33±1.04^{a}	35.17±0.76^{a}	35.33±0.58
50	-	-	-	-	-	-
100	-	8±1.0^{d}	-	-	-	-
250	-	10.33±0.57^{c}	-	-	9.33±0.58^{c}	-
500	11±2.65^{b}	11.67±0.57^{bc}	-	11.17±0.29^{c}	10±1.0^{b}	-
1,000	12.23±0.57^{b}	12.67±0.57^{b}	-	12.17±0.29^{b}	12±0.5^{b}	-
10% DMSO	-	-	-	-	-	-
Susceptibility	Sensitive	Sensitive	Resistant	Sensitive	Sensitive	Resistant

The zone of inhibition was converted to susceptibility rating as follows: resistant, ≤6 mm; susceptible, sensitive, ≥12 mm. Values followed by the same superscript letter in a column did not differ statistically significantly (significance means $p < 0.05$); TC = oxytetracycline; DMSO = dimethyl sulfoxide

Table 2: Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) for *Catunaregam tomentosa* fruit and leaf extracts that had antibacterial activity

Source tissue of extract	*Staphylococcus aureus*		*Bacillus subtilis*	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
Fruit	1,000	nd	1,000	nd
Leaf	1,000	nd	1,000	nd

Nd = not detectable

Minimum inhibitory and minimum bactericidal concentrations

As shown in Table 2, it was found that the two bacterial strains tested (*B. subtilis* and *S. aureus*) were sensitive to both extracts, and the MIC values of these extracts were 1,000 µg/mL. Unfortunately, the extracts did not inhibit the growth of bacteria in testing for MBC, so no MBC values were determined.

Membrane granularity and integrity

Actions of the *C. tomentosa* extracts on bacterial populations, in terms of indicators of granularity and membrane permeability, were investigated from FACS profiles. Flow cytometric profiles, using PI staining to obtain one coordinate and side scatter as the other coordinate, are shown in Figures 1–4. The *C. tomentosa* extracts were used at various concentrations and various exposure times. The results showed that both bacterial strains responded to treatment by the *C. tomentosa* extracts (Table 3 and Table 4). At 3 h of incubation with extract from the leaves of *C. tomentosa,* as the extract concentration increased (0.5 × MIC to 1 × MIC to 2 × MIC), the bacterial population continuously showed changes. First, granularity was lost, followed by loss of membrane permeability, resulting in 3.9 to 16.4 to 25.7 % death rates of *B. subtilis,* and 2.7 to 5.5 to 21.0 % death rates of *S. aureus* at the three MICs, respectively. When the exposure time was 6 h, the *B. subtilis* and *S. aureus* populations again responded continuously (2.1 to 11.5 to 20.5 % death rates, and 8.2 to 11.7 to 34.1 % death rates, respectively, for the MICs). *S. aureus* responded to the leaf extract in a manner dependent on the dose and exposure times, whereas *B. subtilis* responded in a dose-dependent manner with little effect from various exposure times. On treatment with the fruit extract of *C. tomentosa*, increasing the doses from 0.5 × MIC to 1 × MIC to 2 × MIC induced *B. subtilis* death both at 3 h and 6 h of incubation (1.2 to 4.8 to 21.5 %, and 1.6 to 3.2 to 20.1 %, respectively), whereas continuous responses involving the mortality of *S. aureus* were observed at 6 h of incubation (23.7 to 25.2 to 45.7 %, for the three MICs, respectively). Both bacterial strains responded to fruit extracts in a dose-dependent manner. The patterns of bacterial response were identified as follows: Injured cells (lost granularity) and dead cells (cells that initially lost granularity followed by lost membrane permeability) could be seen in the upper left quadrant (PI-negative, SSC-positive) and in the upper right quadrant (PI-positive, SSC-positive), respectively, whereas cells that lost membrane permeability were positioned in the lower right quadrant.

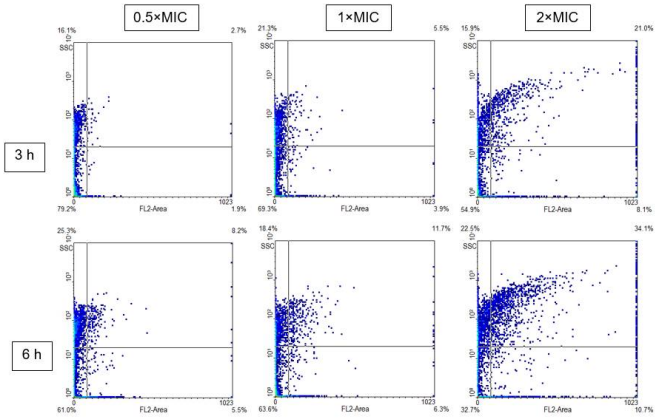

Figure 1: Propidium iodide (PI) staining density plot for the *Staphylococcus aureus* population treated with a leaf extract of *Catunaregam tomentosa* for 3 h and 6 h. MIC, minimum inhibitory concentration. Double negative cells indicate live cell population, PI (or FL2-A) positive cells indicate the membrane-damaged population, Side scatter (SSC) positive cells indicate the lost-granularity population and double positive cells indicate death population

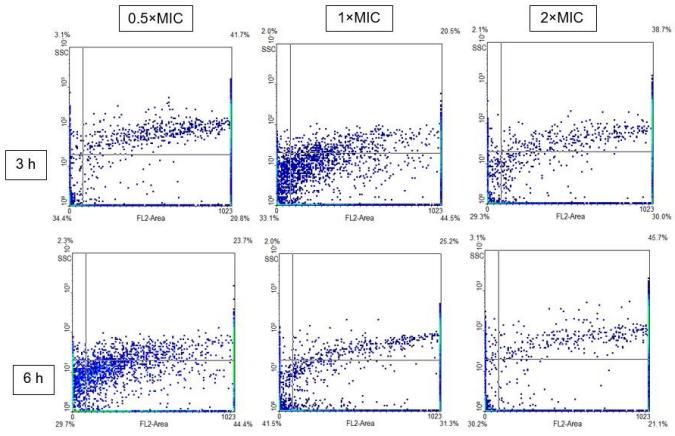

Figure 2: PI staining density plot for the *S. aureus* population treated with a fruit extract of *C. tomentosa* for 3 h and 6 h. Abbreviations and description of all panels are given in Figure 1 legend

Table 3: Fractions of *Staphylococcus aureus* population (in percent) after exposure to extracts of. *Catunaregam tomentosa* at 3 h and 6 h of incubation

Subpopulation of cells	0 h			3 h			6 h	
	Control	*OTC*	*0.5 MIC*	*MIC*	*2 MIC*	*0.5 MIC*	*MIC*	*2 MIC*
Leaf extracts								
Non-responder (viable)	99.9	94.1	79.2	69.3	54.9	61	63.6	32.7
Membrane-damaged	-	0.9	1.9	3.9	8.1	5.5	6.3	1.7
Injured (lost granularity)	-	4.6	16.1	21.3	15.9	25.3	18.4	22.5
Dead	0.1	0.3	2.7	5.5	21	8.2	11.7	34.1
Total responders	0.1	5.8	20.7	30.7	45	39	36.4	58.3
Fruit extracts								
Non-responder (viable)	97.5	96.9	34.4	33.1	29.3	29.7	41.7	30.2
Membrane-damaged	1.7	0.6	20.6	44.5	30	44.4	31.3	21.1
Injured (lost granularity)	0.3	0.2	3.1	2	2.1	2.3	2	3.1
Dead	2.5	3.1	41.7	20.5	38.7	23.7	25.2	45.7
Total responders	0.1	5.8	65.4	67	70.8	70.4	58.5	69.9

MIC = minimum inhibitory concentration; OTC = oxytetracycline

Table 4: Fractions of *Bacillus subtilis* population (in percent) after exposure to extracts of *Catunaregam tomentosa* at 3 h and 6 h of incubation

Subpopulation of cells	0 h			3 h			6 h	
	Control	*OTC*	*0.5 MIC*	*MIC*	*2 MIC*	*0.5 MIC*	*MIC*	*2 MIC*
Leaf extracts								
Non-responder (viable)	80.3	95.5	73.6	45.3	37	75.9	60.3	46.1
Membrane-damaged	18	3.8	20.3	32.1	9.7	20.8	18.8	8.8
Injured (lost granularity)	0.4	0.3	2.1	6.2	27.7	1.2	9.4	24.6
Dead	1.3	0.3	3.9	16.4	25.7	2.1	11.5	20.5
Total responders	19.7	4.4	26.3	54.7	63.1	24.1	39.7	53.9
Fruit extracts								
Non-responder (viable)	62.9.3	58.2	78.7	59.6	39.8	65.5	67.2	45.5
Membrane-damaged	18	20.9	19.7	34.8	34.8	32.4	29.1	31.1
Injured (lost granularity)	9.8	10.6	0.4	0.8	4	0.5	0.5	3.3
Dead	8.7	10.3	1.2	4.8	21.5	1.6	3.2	20.1
Total responders	19.7	4.4	21.3	40.4	60.3	34.5	32.8	54.5

MIC = minimum inhibitory concentration; OTC = oxytetracycline

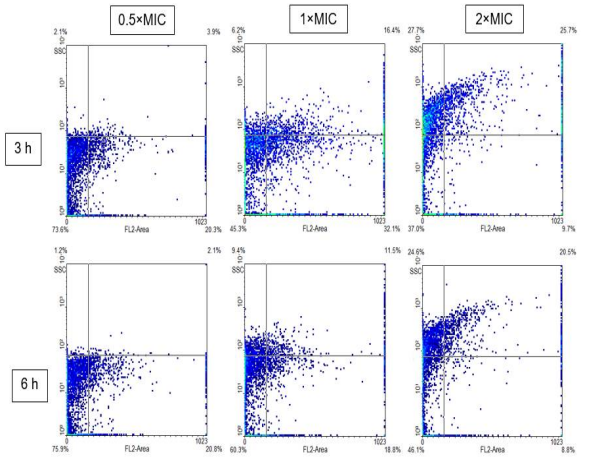

Figure 3: PI staining density plot for *B. subtilis* population treated with leaf extracts of *C. tomentosa* for 3 h and 6 h. Abbreviations and description of all panels are defined in Figure 1 legend

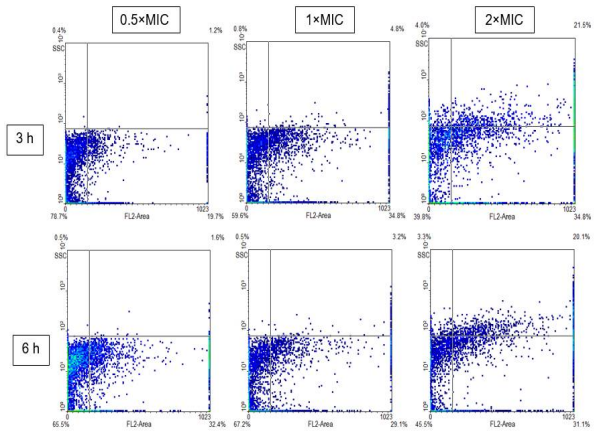

Figure 4: PI staining density plot for *B. subtilis* populations treated with a fruit extracts of *C. tomentosa* for 3 h and 6 h. Abbreviations and description of all panels are defined in Figure 1 legend

DISCUSSION

In this study, the leaf and fruit extracts of *C. tomentosa* displayed upper-moderate to strong antibacterial activities. The inhibition zones of bacteria exposed to *C. tomentosa* extracts indicated that these extracts inhibited the growth of *B. subtilis* and *S. aureus*. Extract doses of 500 – 1,000 µg/disc gave satisfactory clear zone sizes (8 – 12 mm). Although *C. tomentosa* has earlier been reported by Wutithamawech *et al* [6] as a treatment for bacterial infectious symptoms used as a root extract, the root extract did not inhibit the growth of *S. aureus* that was resistant to the extract.

However, in our study, significant differences were observed between extracts from various *C. tomentosa* plant tissues, which had antibacterial activities against *B. subtilis* and *S. aureus*. The results suggested that *B. subtilis* and *S. aureus* were susceptible to fruit and leaf extracts of *C. tomentosa* at 500 – 1,000 µg/disc. Thus, this is the first time that the antibacterial activity of *C. tomentosa* has been documented by direct observations of bacterial populations. The leaf extract was more potent against *B. subtilis* than the fruit extract, whereas the extract activities against *S. aureus* had the reverse order. Unfortunately, a stem bark extract of *C. tomentosa* did not exhibit any antibacterial activity in the current study. The root of this plant has been prescribed in Thai traditional medicine to treat infectious diseases such as stomatitis and ulcer [6], but the current study is the first to report the efficacy of fruit and leaf extracts against the growth of pathogenic bacteria.

Medicinal plants have been traditionally used for the treatment of diseases caused by microorganisms, and the extracts and bioactive compounds from medicinal plants target a wide range of infections in holistic health care. Research on plant-derived substances, which confirmed their activities and determined their specific mechanisms of action against pathogenic organisms, has been widely pursued [8,9]. In the current study, the response patterns of bacterial populations to plant extracts showed changes in granularity and membrane permeability. Time and dose dependence of the responses were assessed, to clarify how the *C. tomentosa* extracts inhibited the growth of these pathogenic bacteria. We observed the bacterial death rates and the changes in granularity and membrane permeability using FACS.

FACS profiles showed that both extracts were active against bacteria, with consistent trends in the death rates. The granularity of the bacteria was lost and the bacterial membranes were damaged by the tested treatments. Most studies have suggested that plant extracts inhibited the growth of bacteria by damaging the cell wall and the cell membrane, thus increasing membrane permeability and causing death of the bacteria [8-10]. By using FACS, we found that increasing the extract dose caused further loss of granularity, and of intracellular functions, subsequently inducing cell membrane injuries, and eventually leading to cell death. Although the outer membrane of a bacterium plays an important role in protecting the cell, permeability of the membrane of Gram positive bacteria could allow a drug to infiltrate into the cell without its disruption [11].

Thus, because the cell first lost granularity, the plant extracts might have played an important role as DNA-damaging agents that were able to penetrate through the cell membrane, and subsequently induce activation of autolysis-induced proteins (i.e., autolysin). The action of autolysin could cause membrane digestion leading to death, indicating programmed death of the bacteria [12]. The patterns of bacterial responses to plant extracts have been explored in recent years, and in one study [10], the cell integrity was lost followed by cytoplasmic content leaking out of the cells and nuclear areas.

However, the mechanisms observed in our current study included changes to granularity and membrane permeability, so that the *C. tomentosa* extracts might act by inducing programmed death in the targeted bacteria. The population of injured cells may be crucial if cell recovery becomes possible [13], as in the development of microbial resistance.

Description of the mode of the action of these extracts could stimulate the search for new antibiotics and the exploration of programmed cell death pathways in bacteria. Multiple mechanisms may be involved, such as loss of granularity and damage to the cell membrane. This is the first report documenting both intracellular changes and outer membrane loss in bacteria exposed to plant extracts. Because the physiological roles of genes involved in programmed cell death are not precisely known for the tested bacteria, the proteins involved in the pathways of bacterial death could be studied in further experiments for a more detailed understanding of how they are involved in the process of programmed cell death.

CONCLUSION

C. tomentosa extracts are potential sources of antibacterial agents against various pathogenic bacteria. The antibacterial effects of the extracts include programmed death of bacteria, starting with granularity changes and DNA damage (injured cells), followed by permeability changes in membranes (membrane-damaged cells), and eventually leading to death. Programmed cell death-mediated proteins should be investigated in future experiments.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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