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TILIA CORDATA: A POTENT INHIBITOR OF GROWTH AND BIOFILM FORMATION OF BACTERIAL CLINICAL ISOLATES

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ABSTRACT

Residents and herbalists residing in Lebanese mountain areas, noticed that tea preparations of *Tilia cordata*, a tree that commonly grows there, alleviated symptoms of respiratory tract and throat infections. This study aimed at determining whether *Tilia cordata* actually was capable of inhibiting the growth of various local clinical bacterial isolates, with particular interest on those that commonly caused respiratory tract infections. Using the agar diffusion method, the results showed that Gram positive bacteria were mainly sensitive to low concentrations of the aqueous extract of *Tilia cordata*'s flower. Bract aqueous extracts did not inhibit any of the tested isolates. Using the agar dilution method, it was noted that the methanolic flower and bract extracts significantly inhibited the growth of the tested bacterial isolates when the ratios of extracts to media were 0.05 and 0.1. All the isolates were, however, completely inhibited when that ratio was 0.15. The methanolic extract of the flower, at a lower concentration, was also capable of inhibiting the ability of the isolates to form biofilms. The active ingredients of *Tilia cordata*, if identified, purified, and evaluated for their safety, can prove to be potent antibacterial agents.

KEYWORDS: Antibacterial compounds; Bacterial infections; Herbalism; Medicinal plants; Plant extracts; *Tilia cordata.*

INTRODUCTION

Resistance against antimicrobial agents has become a common phenomenon. Microbes can accumulate spontaneous mutations and acquire traits through genetic recombination that would render them resistant to an array of antimicrobial agents. Moreover, the rise in the use

and abuse of antimicrobials, especially antibiotics, has contributed to magnify the problem. The acceleration of resistance, in various microbial groups, has created a widespread concern over their management. The World Health Organization (WHO) admitted that antimicrobial resistance has become a global health emergency that will seriously jeopardize progress in modern medicine and alerted that there was an urgent need for more investment in research and development of newer antibiotic to fight these resistant and life-threatening organisms.[1] With research becoming more oriented towards finding newer antimicrobials, medicinal plants always seemed to be an appealing option.

One such plant is *Tilia cordata,* a tree belonging to the family *Malvaceae.* It is deciduous and fast growing and grows 15-30 m in height, with a trunk of up to 1m in diameter. Its bark is smooth, gray, and fibrous, while its leaves are alternate, stalked, mostly hairless, blade heart-shaped (cordate), with an abruptly tapered long tip and unequal-sided base. The *T. cordata* flowers have five petals and sepals, are yellowish to white, strong scented and are produced in clusters of five to eleven in early summer with a large pale colored bract.[2]

The aqueous extracts of *T. cordata* flowers, were reported to have in vitro stimulatory effects on lymphocyte proliferation.[3,4] In vivo data also showed that *Tilia europea* had sedating effects on mice,[5] and *T. cordata* had therapeutic properties for irritated buccal membranes.[6] *Tilia* species were also found to cause vasodilation and to reduce diastolic pressure when injected in rabbits.[7]

On the other hand, the *T. cordata* bract oil was shown to have inhibitory effects on the Gram positive bacteria: *Staphylococcus aureus, Sarcina lutea* and *Bacillus cereus*.[8] The *T. cordata* methanolic extracts were also reported to inhibit the growth of *Listeria ivanovi*,[9] *Streptococcus aureus, Bacillus subtilis, Escherichia coli*,[10] and the foodborne pathogens: *Salmonella* Typhimurium, *S. aureus* and *Vibrio parahaemolyticus*.[11] Moreover, the methanolic extracts, infusions and hydrosols of *Tilia vulgaris* were also demonstrated to have antibacterial properties as they were effective against *Klebsiella pneumoniae, Morganella morganii,* and *B. cereus*.[12]

In traditional medicine, Lebanese mountain residents and herbalists noticed that tea preparations of *T. cordata* flowers and bracts alleviated symptoms of throat and respiratory tract infections. The aim of this study was to determine whether aqueous and methanolic extracts of *T. cordata* exhibited antibacterial and anti-biofilm properties against clinically

significant bacterial isolates, with particular interest on those that commonly caused respiratory tract infections.

MATERIALS AND METHODS

Source of plant

Dried samples of bracts and flowers of *T. cordata* were obtained from Bteghrine, a village in the Maten district of the Mount Lebanon Governorate of Lebanon.

Bacterial isolates

The bacterial isolates used in the study were clinical isolates courteously provided by the Clinical Microbiology Laboratory of the Lebanese American University Medical Center-Rizk Hospital (LAUMC-RH). Namely, the isolates were the following: 3 methicillin resistant *S. aureus* (MRSA), 2 methicillin sensitive *S. aureus* (MSSA), 2 *Enterococcus faecalis* (*E. faecalis*) strains, 1 *Hemophilus influenzae* (*H. influenzae*) strain, 2 extended spectrum beta lactamase producing *Escherichia coli* (ESBL *E. coli*), 2 non-ESBL producing *E. coli* (Non-ESBL *E. coli),* 2 ESBL producing *Klebsiella pneumoniae* (ESBL *K. pneumoniae),* 2 non-ESBL producing *K. pneumoniae* (Non-ESBL *K. pneumoniae*), 3 *Acinetobacter baumannii* (*A. baumanii*) and 2 *Pseudomonas aeruginosa (P. aeruginosa*).

Definitive identification of the bacterial isolates

The identity of the isolates was reconfirmed by using standard methods.[13] The identification of members of the family *Enterobacteriaceae* was also done using the API 20E kits (Biomerieux), while the identification of the non-fermentative bacteria was also done using the RapID NF Plus kits (Thermo Fisher Scientific).

Preparation of the aqueous extracts

The air-dried bracts and flowers were boiled separately in water, in a ratio of 1:10 w/v, until the liquid became thick.

Preparation of the methanolic extracts

The air-dried bracts and flowers were blended with absolute methanol, in a ratio of 1:10 w/v, and the mixtures were transferred to Erlenmeyer flasks and kept in an orbital shaker for 7 days. The supernatants were filtered using millipore vacuum filtration.

Standardization of the bacterial isolates

A suspension of each of the bacterial isolates, to be used in the tests, was adjusted to a turbidity matching that of a 0.5 McFarland standard, equivalent to 1.5×10^8 CFU/ml. as recommended.[14]

Well agar diffusion method

The antibacterial activity of the aqueous extracts was assessed by the well agar diffusion method.[14] Mueller-Hinton agar (MHA) plates were prepared and seeded with the standardized inoculum of each tested organism. Single 12 mm wells were introduced using a cork borer in each of the seeded plates. The wells in 2 seeded plates of each organism tested, were filled with 200 µL and 300 µL of the aqueous extract respectively. The plates were incubated at 35 °C for 24 hours after which the diameters of the zones of inhibition of growth were measured. The reported result was the average of at least 3 diameter readings of each plate. The test for each organism was done using the bract and flower extracts separately.

Agar dilution method

The antibacterial activity of the methanolic extracts was assessed by the agar dilution method as recommended.[15,16] MHA agar was prepared and poured in plates to use as a control. Other MHA agar flasks were prepared and placed in a 70 °C water bath. The methanolic extract of the flowers and bracts were added to the different MHA Erlenmeyer flasks at 0.05, 0.10, and 0.15 ratios of methanolic extract volume to MHA agar volume respectively. The flasks were left at 70 °C for 15 minutes to evaporate the methanol from solution and then poured into petri dishes. The standardized inoculum was then used to seed each of the prepared plates. The plates were then incubated for 24 hours at 35 °C after which any growth on the surface was reported.

Assessment of the methanolic flower extract's interference with biofilm formation

Preparation of the methanolic flower extracts in wells

The initially prepared stock solution of the methanolic flower extract was diluted with methanol in a tailored manner through which final diluted concentrations corresponded to concentrations that were sub-inhibitory to each bacterial isolate. 200 µL of the diluted extracts were then loaded in 96 well, flat-bottom tissue culture plates and left to dry in the incubator for 3 days at 35°C, under aseptic conditions, until the methanol got evaporated from the wells.

Detection of biofilm formation

Bacterial isolates were freshly streaked then used to inoculate 10 ml of trypticase soy broth (TSB). The inoculated TSB tubes were then incubated for 24h at 35 °C. After that, 200 µL of the inoculated TSB were loaded into wells after having the methanol from the flower extract evaporated. The loaded plates were then left for 24h in an incubator at 35 °C to allow for biofilm formation. After the incubation period, wells were emptied from their contents through gentle tapping and washed with 1X phosphate buffered saline (PBS). Crystal violet was used to stain any biofilms that may have formed in the wells. This was followed with a washing phase with 1X PBS. The optical densities (O.D.s) of the wells were later determined by using an ELISA plate reader at a wavelength of 570 nm.

Effect of the flower extract on biofilm formation

For each bacterial isolate, 8 wells were used for each of the following categories: control wells, wells containing the dried flower extract, wells containing the dried flower extracts and inoculated TSB, and wells containing inoculated TSB only. After the incubation period, wells were emptied from their contents through gentle tapping and washed with 1X phosphate buffered saline (PBS). Crystal violet was used to stain any biofilms that may have formed in the wells. This was followed with a washing phase with 1X PBS. The optical densities (O.D.s) of the wells were later determined by using an ELISA plate reader at a wavelength of 570 nm. The reported O.D.s represent the averages of the 8 readings for each test performed.

RESULTS

Antibacterial effects of the aqueous extracts

As shown in Table 1, the aqueous flower extract inhibited the growth of all the Gram positive bacterial strains tested (MRSA, MSSA, and *E. faecalis*) and one Gram negative bacterium: *H. influenza*. No zones of inhibition of growth were detected while using the aqueous bract extract for any of the isolates tested. No significant differences were observed when 200µL and 300 µL of the aqueous extracts were loaded in the wells (Table 1).

Table 1: Average diameters of the zones of inhibition of growth using the *Tilia cordata* aqueous extracts.

	Diameter of the zone of Inhibition of growth (mm)			
Volume added	**200µl**		**300 µl**	
Bacteria	**Flower**	**Bract**	**Flower**	**Bract**
MRSA isolate 1	20 mm	0	20 mm	0
MRSA isolate 2	21 mm	0	20 mm	0
MRSA isolate 3	19 mm	0	19 mm	0
MSSA isolate 1	20 mm	0	20 mm	0
MSSA isolate 2	17 mm	0	18mm	0
E. faecalis isolate 1	19 mm	0	20 mm	0
E. faecalis isolate 2	19 mm	0	16 mm	0
H. influenzae	20 mm	0	22 mm	0
ESBL *E. coli* 2 isolates	0	0	0	0
Non-ESBL *E. coli* 2 isolates	0	0	0	0
ESBL *K. pneumoniae* 2 isolates	0	0	0	0
Non-ESBL *K. pneumoniae* 2 isolates	0	0	0	0
P. aeruginosa 3 isolates	0	0	0	0
A. baumanii 3 isolates	0	0	0	0

Antibacterial effects of the methanolic extracts

Using the agar dilution method, the flower methanolic extracts totally inhibited the growth of all the Gram positive and Gram negative bacterial isolates tested, at a ratio of 0.15 of extract volume to agar volume. A similar result was also noted for the methanolic bract extract except for one *K. pneumoniae* isolate. Significant inhibition or decrease in confluency of growth was also detected when the ratios of extract volume to agar volume were less, at 0.10 and 0.05 (Table 2).

Table 2*:* Growth on the plates containing the *Tilia cordata* methanolic extracts.

	Growth after 24 hours of incubation					
Ratio of extract to MHA	**0.15**			**0.10**	**0.05**	
Bacteria	**Control**	**Flower**	**Bract**	**Flower**	**Flower**	**Bract**
MRSA isolate 1	+++	-	-	-	-	+++
MRSA isolate 2	+++	-	-	-	-	+++
MRSA isolate 3	+++	-	-	-	-	+++
MSSA isolate 1	+++	-	-	-	-	+++
MSSA isolate 2	+++	-	-	-	-	+++
E. faecalis isolate 1	+++	-	-	-	-	+++
E. faecalis isolate 2	+++	-	-	-	+	+++
H. influenzae	+++	-	-	-	-	+
ESBL *E. coli* isolate 1	+++	-	-	++	+++	+++
ESBL *E. coli* isolate 2	+++	-	-	+	++	+++

Non-ESBL *E. coli* isolate 1	+++	-	-	++	+++	+++
Non-ESBL *E. coli* isolate 2	+++	-	-	-	-	++
ESBL *K. pneumoniae* isolate 1	+++	-	++	++	++	+++
ESBL *K. pneumoniae* isolate 2	+++	-	-	+	+++	+++
Non-ESBL *K. pneumoniae* isolate 1	+++	-	-	++	+++	+++
Non-ESBL *K. pneumoniae* isolate 2	+++	-	-	-	+++	+++
P. aeruginosa isolate 1	+++	-	-	-	-	++
P. aeruginosa isolate 2	+++	-	-	-	-	++
P. aeruginosa isolate 3	+++	-	-	-	-	++
A. baumanii isolate 1	+++	-	-	-	+	+++
A. baumanii isolate 2	+++	-	-	-	+	+++
A. baumanii isolate 3	+++	-	-	-	+	+++

+++: Confluent growth; ++: Moderate growth; +: Minimal Growth; -: No Growth.

Biofilm Inhibition by the flower methanolic extract

The optical densities of the wells from the testing of the effects of the methanolic extract on the formation of bacterial biofilms are displayed in Table 3. The flower extract was clearly able to inhibit biofilm formation in almost all of the bacterial isolates tested, being Gram positive or Gram negative bacteria.

Table 3: Average optical density (O.D.) readings at a wavelength of 570 nm of the different wells.

Bacterial Isolates	**Average O.D. reading of the wells containing**			
	Control	Flower Extract	Bacteria+ Flower Extract	Bacteria
Gram Positive				
MRSA Isolate 1	0.109230	0.294796	0.321730	0.239133
MRSA Isolate 2	0.109230	0.498811	0.523747	0.350000
MRSA Isolate 3	0.179463	0.427752	0.299717	0.402284
MSSA Isolate 1	0.109230	0.824557	0.816398	0.322349
MSSA Isolate 2	0.109230	0.713943	0.916231	0.445732
E. faecalis urine isolate 1	0.109230	0.691874	0.572495	0.279826
E. faecalis urine isolate 2	0.109230	0.397397	0.619819	0.696305
Gram Negative				
ESBL *E. coli* isolate 1	0.109230	1.474827	1.299552	0.359582
ESBL *E. coli* isolate 2	0.109230	1.570090	1.550789	0.487658
Non-ESBL *E. coli* isolate 1	0.109230	1.350906	1.393599	0.359582
Non-ESBL *E. coli* isolate 2	0.109230	0.481004	0.481868	0.683819
ESBL *K. pneumoniae* isolate 1	0.109230	2.081927	1.675638	0.495907
ESBL *K. pneumoniae* isolate 2	0.109230	1.668504	1.985758	0.337286
Non-ESBL *K. pneumoniae* isolate 1	0.109230	2.285590	2.711046	0.289036
Non-ESBL *K. pneumoniae* isolate 2	0.109230	1.008697	1.027723	0.195237
A. baumanii isolate 1	0.109230	0.952377	1.120057	0.438730
A. baumanii isolate 2	0.109230	0.992696	0.889144	0.179017

A. baumanii isolate 3	0.109230	1.033660	1.198669	0.175454
P. aeruginosa isolate 1	0.109230	0.348422	1.242858	1.606758
P. aeruginosa isolate 2	0.109230	0.712539	0.667588	0.249042
P. aeruginosa isolate 3	0.109230	0.572029	1.599070	2.436883
A. baumanii isolate 1	0.109230	0.952377	1.120057	0.438730
A. baumanii isolate 2	0.109230	0.992696	0.889144	0.179017
A. baumanii isolate 3	0.109230	1.033660	1.198669	0.175454

DISCUSSION

The fact that the aqueous flower extract exhibited antimicrobial activity against isolates of MRSA, MSSA, *E. faecalis*, and *H. influenzae* was consistent with the traditional use of *T. cordata* tea preparations for treatment of throat and respiratory tract infections. Although there was no significant difference between the diameters of the zones of inhibition of growth when 200 µL or 300 µL of the aqueous flower extract were used, increasing the volume of the extract loaded in the wells more, would probably have increased the size of the diameters of these zones to a substantial extent. The fact that many of the Gram negative isolates, were not sensitive to the aqueous flower extract and none were sensitive to the aqueous bract extract, did not, however, imply that *T. cordata* did not possess antibacterial properties, but suggested that many of the antibacterial components in *T. cordata* may not have been solubilized in water, may have been destroyed during boiling, or may not have diffused properly through the agar.

This is further elaborated by the results of the methanolic extracts, using the agar dilution method, which showed that methanol was an excellent solvent for the extraction of antibacterial compounds present in *T. cordata*. All the Gram positive and Gram negative strains tested were inhibited by the methanolic flower extract, when the ratio of extract volume to the agar volume was 0.15. This was also true for the methanolic bract extract at the same ratio except for the one isolate of *K. pneumoniae* which showed only less confluent growth. At a ratio of 0.10 of flower extract volume to agar volume, only isolates of *E. coli* and *K. pneumoniae* exhibited some growth while all other isolates were completely inhibited. Reducing the ratio of the methanolic flower extract volume to that of the agar volume to 0.05, demonstrated complete inhibition of all the *P. aeruginosa*, *H. influenzae*, MRSA and MSSA isolates, and one isolate of each of *E. coli* and *E. faecalis*. Minimal growth was observed for isolates of *A. baumannii* while the other bacteria showed moderate to confluent growth. The results of this study conform with previous reports that acknowledged that Gram positive bacteria were more susceptible to the essential oils, found in plant extracts, than Gram

negative bacteria.[8, 17, 18] The bract methanolic extract at the same ratio lead to minimal growth only in *H. influenzae* while the other isolates showed either moderate or confluent growth.

The growth of bacterial isolates at sub-inhibitory concentrations does not, however, necessarily mean that the *T. cordata* extract was not effective against them. In fact, it has been described that sub-inhibitory concentrations of antimicrobials may exert multiple effects on bacteria which included: altering bacterial patterns of morphology and growth, targeting the bacterial virulence factors, and affecting the bacteria's susceptibility to the host's immune system.[19] Nevertheless, further studies are needed to assess whether this process and such alterations took place.

Clinical isolates of *S. aureus* are known to develop multiple strategies to acquire antibiotic resistance and are thus considered drug fast organisms.[20] An intriguing finding while using the aqueous flower extract of *T. cordata* against the drug-fast MRSA, was the formation of well-defined margins of the zones of inhibition of growth without any bacterial growth within. The absence of small colonies within the zones of inhibition of growth, usually characteristic of the development of resistant strains, suggest that the extracts worked fast in killing the *S. aureus* strains, not giving them even minimal time to develop resistance.

The fact that the flower methanolic extract of *T. cordata* was more potent than that of the bract methanolic extract, in totally inhibiting the growth of many clinical isolates, even at a ratio of extract to medium volume of as low as 0.05 (Table 2), is certainly attributed to the difference in chemical composition of the flowers and bracts. Further studies are needed to verify this explanation.

The ability of the *T. cordata*'s methanolic flower extract to also inhibit the ability to form biofilms by both the Gram positive and Gram negative bacteria is exhibited in Table 3. In many a case, the average O.D values of wells containing the bacteria and the flower extract was less than that of wells containing bacteria only. This indicated that the methanolic extract of the flower was strongly capable of inhibiting the biofilm formation in those isolates. With most of the other isolates, however, the average O.D values of wells containing bacteria and the flower extract is still less than that of wells containing bacteria and others containing the flower extract combined. This indicated that the methanolic flower extract, at sub-inhibitory concentrations, could prevent the biofilm formation by the tested isolates. This is of particular

importance since biofilms are becoming more problematic with regards to their resistance to antibiotics, increased virulence, and ability to be protected from the host's immune system.

It is worthwhile noting that in this study, the finding that *T. cordata*'s methanolic flower extract was capable of inhibiting the biofilm formation by all the strains of *E. coli* tested, conflicted with the result reported by Samoilova and colleagues in 2014 who reported that the extracts of *T. cordata* actually promoted biofilm formation in *E. coli*.[21] A limitation of that study, however, was that it did not take into consideration the *T. cordata* extract's absorbance, which was a contributing to the increase in O.D of the wells containing both the bacteria and extract, beyond that of control wells, thereby giving the impression that *T. cordata* extracts promoted the biofilm formation by *E. coli*.

It is necessary to mention that many of the test organisms used in this study were multiresistant clinical isolates, obtained from patients suffering from hospital associated infections. The clear and promising results reported in this study should be a call to isolate and utilize the active components of *T. cordata* as prospective new and effective antibacterial agents.

CONCLUSION

T. cordata seems to be a powerful medicinal plant since it has proven to be able to inhibit the growth of many clinically significant bacteria that are multiresistant to available antibacterial agents. Consequently, it may be a potential source of new antibacterial medications. the *T. cordata* flower extract also interfered with the process of biofilm formation of the various bacterial isolates at low concentrations. Additional research is required to identify the active components in the *T. cordata* flower, purify them, and assess their safety for consumption by infected patients.

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