

***In-vitro* Antibacterial Activities of Alkaloids Extract from Leaves of *Conocarpus lancifolius* Engl.**

Hayssam M. Ali^{1,3*}, Mohamed Z.M. Salem² and Ahmed Abdel-Megeed^{1,4}

¹ Botany and Microbiology Department, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.

² Forestry and Wood Technology Department, Faculty of Agriculture (EL-Shatby), Alexandria University, Alexandria, Egypt.

³ Timber Trees Research Department, Sabahia Horticulture Research Station, Horticulture Research Institute, Agriculture Research Center, Alexandria, Egypt.

⁴ Plant Protection Department, Faculty of Agriculture (Saba Basha), Alexandria University, Egypt.

(Received: 28 June 2013; accepted: 16 August 2013)

Natural biocidal products produced by plants are the major source of bioactive substances with strong pharmacological action and less cytotoxicity. The antibacterial activities of the alkaloid extracts of the leaves of *Conocarpus lancifolius* Engl. were bioassayed against certain plant and human pathogenic bacteria; *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Agrobacterium tumefaciens* and *Erwinia amylovora*. The inhibition zones were ranged between 10-17 mm and the MICs were between 10 and >200 µg/mL. Alkaloidal extract of *C. lancifolius* was active at all concentrations tested (5-200 µg/mL) against the studied bacteria except *E. amylovora*. The result suggests the applications are also growth promotive and cost effective and non-hazardous in agro-ecosystem. Further work should be done to elucidate the valuable of *C. lancifolius* as a source of antibacterial for human and plant health.

Key words: *Conocarpus lancifolius*, antibacterial activity, alkaloid extract.

The natural plant products, known as botanical pesticides or herbal medicines, have long been used in the control of microorganisms causing plant and human diseases. However, with the employment of synthetic pesticides in agriculture, the use of botanical pesticides has significantly diminished.

Conocarpus lancifolius Engl., one of two species in the genus *Conocarpus*, is a tree in the family Combretaceae native to coastal and riverine areas of Somalia, Djibouti, and Yemen. It is found throughout East Africa, the Arabian Peninsula, and South Asia (Redha *et al.* 2011). *C. lancifolius* is an ornamental plant that flourishes under the semi-arid conditions (Al-Kandari *et al.*, 2009). Most of the research studies focused on its responses to

environmental stress (Redha *et al.* 2012). The titer and type of polyamine accumulated in *C. lancifolius* appeared to be related to the nature, intensity and duration of environmental stress. Understanding its response to drought and salinity stress will assist in the management and longevity of this species (Al-Kandari *et al.*, 2009).

To our knowledge from literature there is little work about the biological activities of *C. lancifolius* which supported the authors to put into practice this study. The medicinal uses are unknown as reported for the literature (Al-Musayeib *et al.* 2012). The methanol extract of *C. lancifolius* demonstrated a noticeable cytotoxic effect as an antiprotozoal activity (Al-Musayeib *et al.* 2012). On the other hand, the other species; *C. erectus* was studied for the biological activity and four defatted methanol extracts of *C. erectus* different parts (leaves, stems, fruits and flowers) showed high free radical scavenging activity

* To whom all correspondence should be addressed.
Tel.: +966563772132; Fax: +966114675833;
E-mail: hayssam77@hotmail.com

toward DPPH radical (Abdel-Hameed *et al.* 2012). Fifteen different phospholipids were detected in leaf extracts by TLC and 6 major fatty acids were identified by GC/MS analyses. The omega-3 fatty acid, linolenic acid was the predominant fatty acid present at 61.32% in the 10% PEG-treated plants and its high concentration may help *C. lancifolius* to adapt to semi-arid conditions in the State of Kuwait (Redha *et al.* 2012). The objective of this study is to evaluate the alkaloidal extract against the growth of some pathogenic bacteria. Implications of this study may highlight the medicinal valuable of *C. lancifolius* in the term of the antibacterial of alkaloidal extract against some human and plant bacterial strains.

In this context, the present study was undertaken in order to find out the effort of this botanical plant extracts on for controlling certain plant and human pathogenic bacteria responsible for many diseases.

MATERIALS AND METHODS

Plant material

Fresh leaves of *C. lancifolius* were collected from the garden of the Faculty of Agriculture, university of Alexandria. The leaves of *C. lancifolius* were picked up from three levels of the tree trunk (Figure 1) in May 2013. Then washed with tap water and dried at room temperature and pulverized into powder with small laboratory mill. The plant was kindly identified and authenticated at the Department of Forestry and Wood Technology, Faculty of Agriculture, Alexandria University.

Preparation of the extract

The fresh leaves were washed, air-dried under shade at room temperature and then milled into powder using a laboratory small mill. The air-dried powder (100 g) was soaked with 200 mL of methanol (80%). After one week of soaking the solution was passed through activated charcoal to remove the chlorophyll and filtrated. After filtration the residue was processed similarly with the same amount of solvent (Salem *et al.* 2012; Salem *et al.* 2013a). The crude methanol extract was concentrated to dryness using a rotary evaporator under reduced pressure at 45°C. The extract was lyophilized and stored at 4°C in the refrigerator until further use.

To precipitate the alkaloids from the CHCl_3 fraction, samples of about 1 g from the lyophilized methanol extract were dissolved in 50 mL of 99% methanol and treated with an equal volume of 1% aqueous HCl then the alkaloids were precipitated by drop-wise addition of 10% NH_4OH (Harborne, 2005; Cannell, 1998). The precipitated was collected by centrifugation (5000 rpm at 4°C for 30 min.) and washed with 1% NH_4OH . The residue was dissolved in a few drops of CHCl_3 to obtain the CHCl_3 fraction that was containing the precipitated alkaloids. The solvents were removed under reduced pressure and the extracts were concentrated under vacuum at 40-60°C. The chloroform fraction obtained was then weighed and prepared for stock solution at a concentration of 200 $\mu\text{g/mL}$ by diluting the crude extract in 99.5% Dimethylsulfoxide (DMSO, Sigma-Aldrich).

Test for alkaloids

The methanol extract (0.5 g) was diluted in 10 mL of acid alcohol, boiled and filtered. Diluted ammonia (2 mL) was added to 5 mL of the filtrate. 5 mL of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 mL of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Dragendorff's reagent



Fig. 1. The three levels of *C. lancifolius* trunk.

to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids. Additionally, the precipitated alkaloids that were found by centrifugation were also treated with same method described previously (Harborne, 2005).

Biological Assays

The antibacterial activity was carried out on the precipitated alkaloids with concentration of 200 µg/mL against three Gram-positive bacteria; *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538; three Gram-negative bacteria: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Serratia marcescens* ATCC 13880 were used in the present study as human pathogenic bacteria. The Gram-negative bacteria; *Agrobacterium tumefaciens* ATCC 33970 and *Erwinia amylovora* ATCC 49946 were used as plant pathogens. Nutrient agar (NA) medium was used for maintenance of the tested bacterial organisms. Mueller Hinton agar (MHA) was used in all bioassays applying the disc diffusion method.

Kirby-Bauer disc diffusion method

The Kirby-Bauer disc diffusion susceptibility test (NCCLS, 1997) method was used to measure the sensitivity of the bacterial strains to the precipitated alkaloids. The tested bacteria with a suspension of 1 mL of 10^5 CFU/mL were spread on the surface of solid media plates. Filter paper discs with 5 mm in diameter were loaded with 20 µL of the precipitated alkaloids and placed on the inoculated plates. The plates were incubated at 37°C for 24 h. The diameters of the inhibition zones (IZs) were measured in millimeters. The biological activities of the precipitated alkaloids were carried out at the concentration of 200 µg/mL against the tested bacterial strains. The IZs obtained were compared with a positive control

(Tetracycline 20 µg/disc) and for the negative control; discs were saturated with 20 µL of DMSO. The experiment was done in triplicate.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined by serial dilution of the precipitated alkaloids (5, 10, 20, 50, 100 and 200 µg/mL) in 96-well micro-plates (Eloff, 1998) by filling all wells with 50 µL sterile Mueller Hinton Broth (MHB) with minor modification. Two wells were used as a sterility and growth control respectively with the sterility control containing only Oxoid® MHB (Sigma-Aldrich), whilst the growth control containing both MHB as well as test organism. After adding 50 µL of the bacterial suspension (10^5 CFU/mL) to each row (except for the sterility control), the micro-plate was covered and incubated at 37°C at 100% relative humidity overnight. 50 µL of a 0.2 mg/mL solution of *p*-iodonitrotetrazolium violet (Sigma-Aldrich) was added to each well to indicate the inhibition of the growth by a clear decrease in color reaction.

Statistical analysis

The results are expressed as mean values $\pm$ standard deviation (SD). Analysis of variance was used to evaluate the significant difference among the means at 0.05 level of probability (SAS, 2001).

Table 1. Quantification of alkaloid extracts from *C. lancifolius* leaves

Level	Sample (g)	Methanol extract (g)	Alkaloids (g/100 g Methanol extract)
1	50	6.672b	27.47a
2	50	6.294b	25.70b
3	50	8.407a	24.52b

Means with the same letter within the same column are not significantly different at 0.05 level of probability

Table 2. Detection of alkaloid by Draggendorff and Mayer reagents in *C. lancifolius* leaves

Level	Draggendorff's reagent		Mayer's reagent	
	Chloroform layer	Precipitated alkaloids	Chloroform layer	Precipitated alkaloids
1	+++	++++	++	++
2	++	++++	+	+++
3	++	++++	+	++

(+) Indicates the intensity of the color formed

Table 3. Diameter of inhibition zones of alkaloidal extract against the growth of some pathogenic bacteria

Level	Bacterial strains							
	<i>B. subtilis</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>S. marcescens</i>	<i>E. coli</i>	<i>A. tumefaciens</i>	<i>E. amylovora</i>
1	13±1.2b	13±0.9a	10±1.3c	14±2.7a	10±0.8b	15±1.4b	14±1.1b	na
2	16±1.3a	13±0.78a	12±1.7b	14±2.1a	15±0.7a	16±1.3a	17±1.12a	na
3	13±1.2b	13±0.89a	14±1.7a	13±1.7b	10±0.8b	16±1.4a	17±1.17a	na
Tet.*	18	17	21	27	20	18	n.t.	n.t.
DMSO	na	na	na	na	na	na	na	na

Means with the same letter within the same column are not significantly different at level of probability 0.05

Inhibition Zone (Values are expressed as mean±SD including disc diameter of 5 mm at 200 µg/mL); na: Not active; n.t. Not tested. * Tetracycline (20 µg/disc).

Table 4. Minimum inhibitory concentrations of alkaloidal extract against the growth of some pathogenic bacteria

Level	Bacterial strains							
	<i>B. subtilis</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>S. marcescens</i>	<i>E. coli</i>	<i>A. tumefaciens</i>	<i>E. amylovora</i>
1	50	20	100	50	100	10	50	> 200
2	20	50	100	50	50	20	20	> 200
3	50	50	50	50	100	20	20	> 200

Values of minimum inhibitory concentrations are expressed as µg/mL

RESULTS AND DISCUSSION

The existing knowledge about the biological activity of extracts from *C. lancifolius* is very limited, so it is important to mention that to the best of our knowledge, this study represents the first report on antibacterial activities for the precipitated alkaloids from *C. lancifolius*. The precipitated alkaloids from the leaves of *C. lancifolius* were 27.47, 25.70 and 24.52 g/ 100 g of methanol extract from the trunk level 1, 2 and 3, respectively (Table 1). On the other hand, the color detection of the alkaloids as shown in Table 2 represents that the precipitated alkaloids had the highest color intensity than in the chloroform layer as measured by Mayer's reagent's and Draggendorff's reagents.

Antimicrobial activity of the precipitated alkaloids of *C. lancifolius* leaves was evaluated by measuring the diameters of the inhibition zones and MICs values of the growth of some human and plant pathogenic bacteria (Tables 3 and 4) within three levels of tree trunk. All the tested bacterial strains were susceptible to precipitated alkaloids of *C. lancifolius* with different degrees

of IZs except *E. amylovora* which observed a resistance to all the concentration used in the present study. However, it was found that, on the basis of IZs, there are varied in the susceptibility of bacteria to plant extracts according to strains and species (Karou *et al.*, 2006). On the other hand, the presence of alkaloids in the extracts has been shown to possess an antimicrobial and antioxidant activities (Erdemoglu *et al.*, 2007; Salem *et al.*, 2013; Salem *et al.*, 2013b). The highest IZs (17±1.12 and 17±1.17mm) were observed against the growth of *A. tumefaciens* by the alkaloidal extract from the levels 2 and 3 of the tree trunk, respectively. The lowest IZs were found against *S. aureus* (10±1.3 mm, level 1) and *S. marcescens* (10±0.8 mm, levels 1 and 3). Additionally, it can be seen that the precipitated alkaloids had a varied IZs within the levels of the trunk against the same bacterium, for example, it observed an IZ (16±1.3 mm) with *B. subtilis* by the level 2 and 13±1.2 mm by the levels 1 and 3.

Table 4 presents the MICs values against the growth of the tested bacterial strains. *B. subtilis* had an MIC at 20 µg/mL (level 2), *B. cereus* (20 µg/mL at level 1), *S. aureus* (50 µg/mL at level 3), *P.*

aeruginosa (50 µg/mL at all levels), *S. marcescens* (50 µg/mL at level 2), *E. coli* (10 µg/mL at level 1), *A. tumefaciens* (20 µg/mL at levels 2 and 3) and *E. amylovora* had MIC value > 200 µg/mL within the all levels. Additionally, it can be observed that *E. coli* is sensitive to the alkaloidal extract by the three levels of the tree trunk. On the other hand, *E. amylovora* was resistant to alkaloids extract at all the levels of tree trunk.

CONCLUSIONS

In conclusion, the current work has led to the alkaloids extract of *C. lancifolius* leaves exhibiting relevant antibacterial activity. The medicinal plants such as *C. lancifolius* can be promising sources of potential antibacterial, and our results are supported this idea. The result of present study can be further exploited for formulating integrated disease management schedule of these plant pathogenic bacteria. It will form the basis for further work and investigation on *C. lancifolius* in the potential discovery of new natural bioactive compounds that can be used as antimicrobial and antioxidant activities for human and plant pathogens.

ACKNOWLEDGMENTS

This project was supported by King Saud University, Deanship of Scientific Research, College of Science Research Center.

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