academicJournals

Vol. 14(4), pp. 292-297, 28 January, 2015
DOI: 10.5897/AJB2014.13942
Article Number: A65F35C49960
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Activity of *Cinnamomum zeylanicum* essential oil and ethanolic extract against extended-spectrum β-lactamase-producing bacteria

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Received 18 July, 2014; Accepted 15 January, 2015

The antibacterial effects of Cinnamon (*Cinnamomum zeylanicum*) essential oil and ethanolic extract against extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Pseudomonas aeruginosa* strains were studied in the present study. The essential oil and ethanolic fraction of *C. zeylanicum* showed significant activities against all tested microorganisms and minimal inhibitory concentration values (MIC) of the essential oil ranged from 0.8 to 20.2 µg/ml. The MIC of ethanolic fraction at 60°C were in the range from 8 to 62.12 µg/ml, although at room temperature showed the highest and lowest activity at 14.5 and 64.11 µg/ml, respectively. The results show by these extracts recommends their potential use against multidrug resistant microorganisms. This study also shows that *C. zeylanicum* could be a potential source of new antimicrobial agents. PCR amplification reaction showed the presence of CTX-M β-lactamases gene in all tested organisms.

Key words: *Cinnamon*, CTX-Mβ-lactam-resistant bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*.

INTRODUCTION

Medicinal plants are natural resources for the preparation of valuable products that can be used in the treatment of various ailments. Plant materials remain an important resource for combating illnesses, including infectious diseases, and many plants have been investigated with the intention to produce novel drugs for the development of new therapeutic agents (Tajkarimi et al., 2010; Sienkiewicz et al., 2013). Thus the emergence of multiple drug resistance of pathogenic organisms has caused an extensive research to find new antimicrobial substances from other sources including plants (Lin et al., 2005; Warnke et al., 2009; Yap et al., 2013). This interest have been triggered due to the increasing frequency of microorganisms that are resistant to common and generally accepted antibiotics which is on the increase. Antibiotic resistance refers to the ability of a microorganism to withstand the effects of an antibiotic. Resistance rate to these drugs is higher in developing compared to developed countries because of the extensive and indiscriminate use of antibiotics over the last few decades (Akram et al., 2007) and people propensity to self-medicate without a prescription from an expert.

Among the wide array of antibiotics, beta (β)-Lactam

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(penicillins, cephamycins etc.) are the most varied and widely used (Bronson and Barrett, 2001). The most common cause of bacterial resistance to β-lactam antibiotics is the production of β-lactamases, enzymes that break the antibiotic structure. The majority of these enzymes have been described in Gram negative bacteria which are responsible for numerous infectious diseases and are generally multidrug resistant. Gram-negative bacteria are more resistant to antibiotics than the Gram-positive bacteria due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Shanab et al., 2004).

Bacterial resistance to β-lactam antibiotics has been attributed to the spread of plasmid-mediated extended spectrum β-lactamases (ESBLs) (Khan et al., 2010; Sienkiewicz et al., 2013). ESBL-producing Enterobacteriaceae and other kinds of bacteria have been reported widely. Infections caused by ESBL-producing bacteria have become a clinical and therapeutic problem because these organisms are resistant not only to β-lactamases but also to many other antimicrobial agents (Velasco et al., 2007). A feasible approach of limiting the transmission of these pathogens is the use of essential oils as alternative or topical agents.

Cinnamon is a plant that belongs to the family of Lauraceae most noted for its bark, which provides the world with the commonly known culinary spice, cinnamon. Cinnamon has medicinal property and has been used to treat gastrointestinal complaints and other ailments (Cao and Anderson, 2011; Varalakshmi et al., 2014) and has been known as a popular remedy. It is reported to exhibit antimicrobial activity (Souza et al., 2007; Mishra et al., 2009; Pritam et al., 2013). Its potential therapeutic roles such as diaphoretic, carminative, antispasmodic, antiseptic, insecticidal and parasiticide properties have also been recognized.

The CTX-M β-lactamases are one of the groups of extended spectrum β-lactamases (ESBLs) identified after the introduction of the broad-spectrum cephalosporins. These enzymes are worldwide spread and 54 different types have already been identified in the last decade. The bla CTX-M genes are located on large plasmids (58-320 kb) that are transformed into *Escherichia coli* and resulted in multiresistant transformants (Soge et al., 2006).

In the present study, essential oil and ethanolic extract of *Cinnamomum zeylanicum* were tested to screen their antimicrobial activity (MIC) against multi-drug resistant *E. coli* (ESBL positive) and *Pseudomonas aeruginosa* strains with verification of CTX-M β-lactamases.

MATERIALS AND METHODS

Plant materials

The essential oil and the powder of *C. zeylanicum* were purchased from the Aroma Trading market (SH pharma), Egypt.

Preparation of plant extract

The bark powder of *C. zeylanicum* (10 g) was refluxed with absolute ethanol (100 ml) for 6 h. The solvents were evaporated at a constant temperature of 60°C until a very concentrated extract was obtained (5 ml). Identification tests for the various chemicals were conducted to test the presence of different chemical constituents.

Preliminary phytochemical analysis

Qualitative phytochemical screening for various chemical constituents including alkaloids, flavonoids, glycosides, phenols, resins, sugars, amino acids, protein, steroids/terpenes and tannins were performed using the crude extract of *C. zeylanicum.* The presence of resins, alkaloids, tannin and proteins was determined according to the methods described by Afaq et al. (1994) and Evans (2002). The presence of amino acids, glycosides, sterols/terpenes, phenols and carbohydrates was demonstrated by the methods of Evans (2002). For the confirmation of the presence of flavonoids, the method of Fornasworth (1966) was used.

Test organisms

The bacterial strains were either reference strain acquired from King Khalid University, Medicine Collage, KSA or clinical isolates from Mansoura University hospital, Egypt. Clinical strains were identified by conventional techniques (Fornasworth, 1966). The isolates studied included Gram-negative bacteria *E. coli* spp. (six strains) and *P. aeruginosa* (two strains). They were tested against their ability to produce β-lactamases enzyme. The isolates studied were resistant to at least one β-lactam antibiotic. Reference bacterial strain was β-lactamase producer *E. coli* ATCC O1577. Antimicrobial susceptibility of these strains was determined by disc diffusion method test. Each strain was routinely sub-cultured, at 37°C, on nutrient agar plate (Difco).

Antibiotic susceptibility testing

Susceptibility testing was performed according to Clinical and Laboratory Standards Institute (NCCLS) recommendations by using microtitre plates containing different dehydrated antibiotics. The initial screening was performed by testing the zone diameter for ceftriaxone, ceftazidime and cefotaxim (Aligiannis et al*.,* 2001) alone and in combination with 4 g/mL clavulanic acid for phenotypic detection of β-lactamases.

Antimicrobial activity test

The antimicrobial activities of the essential oil and the ethanolic plant extract of *C. zeylanicum* were measured *in vitro* against the 9 microbial cultures by using disc diffusion method (Anon, 1997). Filter paper discs (5 mm diameter) were placed on the pre-inoculated agar surface and impregnated with 30 µl of each essential oil or ethanolic fraction. The plates were then incubated at 37°C for 24 h before the diameters of inhibition zones around each disc were measured. All tests were performed twice and the antimicrobial activity was expressed as the mean of inhibition diameters (mm).

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevents visible growth of the bacteria

(Delaquis et al., 2002). One ml from the dissolved essential oil in ethanol and ethanolic extract (100 µg/ml) was used to determine MIC by serial dilution with nutrient broth (Murray et al., 1995). The bacterial cell number was adjusted at 10^6 bacterial cells/ml (0.1 ml inoculum / tube) for the tested strains. All samples were incubated at 37°C during 18-24 h. As control, ethanol was used.

DNA extraction

Bacterial chromosomal DNA was obtained using Maxwell 16 DNA cell purification Kit with automatic DNA extraction machine according to instruction manual. The chromosomal DNA was checked by electro-phoresis in agarose gels, and the concentrations of the different extracts were standardized by spectrophotometric measurements (Sambrook et al., 1989).

Detection of the CTX-M gene

DNA from different *E. coli* strains and *P. aeruginosa* strains was used as the template in a PCR amplification. Amplification reactions were performed in a final volume of 50 µl. Mg^{2+}-free PCR buffer was purchased as a 10× concentrate consisting of 500 mM KCl, 100 mMTris-HCl (pH 9.0), and 1% Triton X-100 (Perkin-Elmer, Roche Molecular Systems, Inc., Nutley, N.J.) with 200 µM (each) dATP, dCTP, dGTP, and dTTP (Perkin-Elmer, Roche Molecular Systems, Inc.). The Mg^{2+} concentration was 2.5 mM, and the primers were used at 0.5 µM each. The primer pair CTX-M-1 (5′-AACACGGATTGACCGTATTG-3′) and CTX-M-2 (5′-TTACAGCCCTTCGGCGAT-3′) was used to amplify the CTX-M-14 gene in the plasmid DNA. Amplification reactions were carried out in T100 thermal cycler (Bio-Rad, Germany), with an initial denaturation (10 min at 94°C) followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C), and extension (2 min at 72°C), with a single final extension of 10 min at 72°C. Aliquots (15 µl) of each sample were subjected to electrophoresis in 1.0% agarose gels. Amplified products were detected after staining with ethidium bromide (50 µg/ml) and photographed with gel documentation system (BioScience, Taiwan).

RESULTS AND DISCUSSION

With the rise in the emergence of various multidrug resistant microorganisms and the scenario worsening through the indiscriminate use of antibiotics, new and/or alternative antimicrobial compounds must be developed to treat common infections (Shakil et al., 2010). New and safe antimicrobial agents are needed to prevent and overcome severe bacterial infections and the problems of bacterial resistance (Sienkiewicz et al., 2013). Plants essential oils and extracts especially cinnamon have been used for many thousands of years, in pharmaceuticals, alternative medicine, and natural therapies (Pritam et al., 2013). It is necessary to investigate this plant scientifically to improve the quality of healthcare.

The initial screening was carried out to test inhibition zones of ceftriaxone, ceftazidime and cefotaxime, and the corresponding inhibitory diameters were 17, 15 and 14 mm, respectively. Thus, it was possible that this strain might produce ESBLs. Moreover, the confirmation test of ESBL production indicated that inhibition zones of cefotaxime and ceftazidime with clavulanic acid were 20 and 17 mm, respectively. The increase of 6 mm between the zones of cefotaxime and cefotaxime combined with clavulanic acid was considered to indicate the production of ESBLs. These results were confirmed with the results of Hongbin et al. (2008) who stated that the increased zone diameter for ceftazidime or cefotaxim tested in combination with clavulanate versus the zone when tested alone was considered indicative of ESBL production from *E. coli* isolates obtained from chicken livers.

Results obtained in the present study reveal that the essential oil and the fractions of the plant extract tested possess potential antibacterial activity against the tested organisms. There are differences in the antimicrobial activities of the essential oil and the ethanolic plant extract as each might possess different compounds. This is in agreement with the report of Khan et al. (2010) which stated that the ability of the oil and extract of some medicinal plants to inhibit bacteria suggested the presence of broad spectrum antibiotic compounds. Schmidt et al. (2006) showed that the essential oil of *C. zeylanicum* is rich in eugenol and cinnamaldehyde which are the two major chemical components that are mainly responsible for its antimicrobial properties. Snyder (1997) stated that eugenol, a phenol compound, inhibits mold and adds flavor and aroma to bakery items. It also possesses antiviral properties *in vitro* while, Ali et al. (2005) confirmed that eugenol and cinnamaldehyde inhibit *H. pylori* growth at a low pH, showing their efficacy in eliminating the bacteria present in the human stomach.

It has been published that various plant extracts have been demonstrated to possess antibacterial activity against microbial pathogens. The antimicrobial activity observed could be due to the varied phytochemicals presents (Aduol et al., 2014). Several metabolites from medicinal plants such as sterols, tannins and alkaloids have previously been associated with antimicrobial activity (Leven et al., 1979). It is necessary to identify the phytochemical component of the local medicinal plants to explain some of the biological activity of certain plant extracts observed.

The phytochemical analysis of ethanolic extract of *C. zeylanicum* showed positive tests at room temperature and at 60°C for the presence of many compounds like resins, sterols /terpenes, tannins, glycosides, alkaloids and flavonoids while reducing sugars, amino acids, phenols and proteins were not detected (Table 1).

The antibacterial activity of cinammon ethanolic extract could be due to the presence of resins, sterols, terpenes, tannins, glycosides, alkaloids and flavonoids. Shuaib et al. (2013) concluded that the resin rich methanolic extracts of *C. myrrha*, *O. turpethum* and *P. Roxburghii* exhibited some degree of antimicrobial activity against Gram-positive (*S. aureus, B. subtilis, M. luteus, E. faecalis*) and Gram-negative bacterial strains (*E. coli, P. aeruginosa, S.*

Table 1. Qualitative test for various phytochemical constituents in ethanolic extract.

Compound	Ethanolic extract (at 60°C)	Ethanolic extract (at room temperature)
Resins	+ve	+ve
Sterols/terpenes	+ve	+ve
Reducing sugers	-ve	-ve
Tannins	+ve	+ve
Glycosides	+ve	+ve
Alkaloid	+ve	+ve
Amino acids	-ve	-ve
Flavonoids	+ve	+ve
Phenols	-ve	-ve
Proteins	-ve	-ve

Table 2. MIC (µg/ml) values of essential oil and ethanolic extract of *C. zeylanicum* studied against multi-drug resistant strains of bacteria.

Strain number	Essential oil	Ethanolic extract (at 60˚C)	Ethanolic extract (at room temperature)
P. aeruginosa 1	20.20	64.00	64.11
P. aeruginosa 2	20.00	62.12	64.00
E. coli 1	0.8	8.22	16.20
E. coli 2	12.70	8.00	14.50
E. coli 3	4.88	32.00	38.35
E. coli 4	4.00	24.80	24.00
E. coli 5	4.00	24.12	22.30
E. coli 6	16.33	30.00	32.11
E. coli ATCC O1577	16.00	32.00	60.00

typhi, S. dysenteriae). Dherbomez et al. (1995), stated that a sterol, 7-amino- cholesterol displayed antibiotic activity against *Saccharomyces cerevisiae*, *S. aureus*, *Enterococcus hirae* and *Bacillus cereus*, while, Cantrell et al. (2001), investigated the importance of terpenes and terpenoids for their antimicrobial activity. Haslam (1996) reported that a wide range of anti-infective actions have been assigned to tannins. Iyengaroside-A (2), a glycoside isolated from the methanolic extract of the marine green alga *Codium iyengarii* has been reported to show bactericidal activity (Ali et al*.,* 2002). Bromotyrosine alkaloids have demonstrated high antimicrobial activity against a number of Gram-positive organisms, including Mycobacteria and Staphylococci (Pick et al*.,* 2006). Flavonoids have been found to show *in vitro* antimicrobial activity against a wide range of bacteria (Cowan, 1999).

The ethanolic extract of cinammon showed significant activity against the investigated bacterial strains, which is promising. It is interesting to note that the extract is not pure compounds and in spite of it, antimicrobial results were obtained, which only suggests the potency of this extract. The potential for developing antimicrobials from plants is rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose without any adverse effects that are often associated with synthetic compounds; hence isolation and purification of phytoconstituents from our plant may yield significant novel antimicrobials.

The essential oil and ethanolic fraction of *C. zeylanicum* showed significant activities against all the microorganisms and the values of MICs for the essential oil ranged from 0.8 (*E.coli* no.1) to 20.2 µg/ml (*P. aeruginosa* no.1) (Table 2). On the other hand, MICs of the ethanolic fraction at 60^{o}C showed highest activity against *E. coli* no.2 (8 µg/ml) while the lowest activity was against *P. aeruginosa* no.1 (64.00 µg/ml). The MICs of ethanolic fraction at room temperature showed the highest activity against *E. coli* no.2 (14.5 µg/ml) and the lowest in *P. aeruginosa* no.1 (64.11 µg/ml). Ethanol was used as a control which did not show any inhibitory activity against each bacterial species.

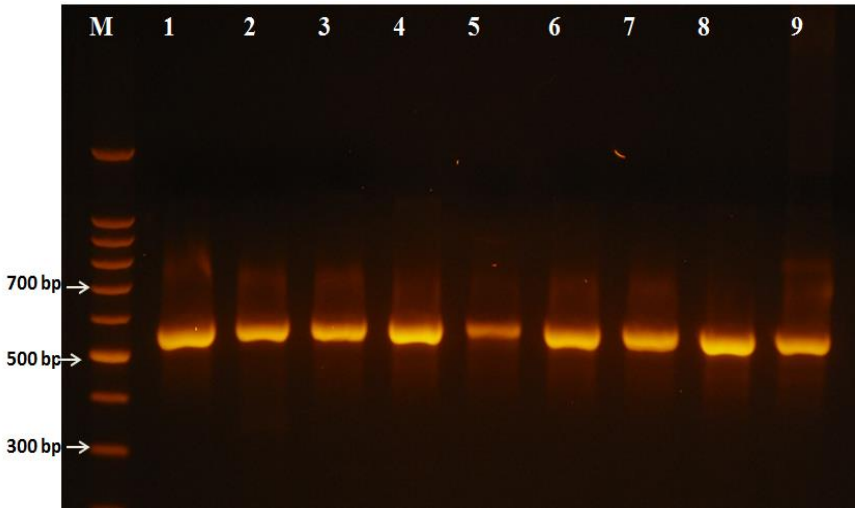

Figure 1. PCR amplification products of CTX-M gene. From left to right; DNA marker: 100bp DNA ladder; lanes 1-6, *E. coli* isolates; lanes 7 and 8 *P. aeruginosa* isolates; lane 9, positive control *E. coli* ATCC O1577 strain containing of CTX-M gene of about 550 bp.

Tests have shown that cinnamon oil have strong antimicrobial effects against the most bacteria and fungi, even more than the cinnamon ethanolic extract. Gupta et al. (2008), showed that cinnamon oil was effective against 10 examined bacteria, including *S. aureus*, *Listeria monocytogenes*, and *E. coli* while cinnamon extract was only effective against most of the food-borne microorganisms. In another study which examined the antibacterial effects of various plant essential oils, cinnamon oil showed maximum activity against the gram positive bacteria *B. subtilis* and *K. pneumoniae* and the gram negative bacteria *P. aeruginosa* and *E. coli* 18 (Ankri and Mirelman, 1999).

There has been a dramatic increase in the number of organisms reported in the literature that produce CTX-M-β-lactamases. This class of β-lactamases has been recognized worldwide as an important mechanism of resistance to oxyimino-cephalosporins used by gram-negative pathogens (Bonnet, 2004) (Figure 1).

To overtake the technical difficulties encountered in molecular detection and characterization of these β-lactamases simultaneous amplification PCR methods have been successfully developed (Colom et al., 2003). The specific fragments at 550 bp was clearly separated and visualized by gel electrophoresis. *E. coli* isolates harboring ESBLs are significantly more frequently found to be resistant to other antibiotics, in particular fluro-quinolones. Due to the increased complexity of β-lactam resistance in Gram-negative organisms, the key to effective surveillance is the use of both phenotypic and genotypic analyses (Eisner et al., 2006). This high ESBL frequency may have been caused by the excessive use of broad-spectrum antibiotics in our hospital, together with a lack of attention to laboratory screening of ESBL production by clinical isolates. On the other hand, the high rate of ESBL production could possibly be due to the spread of 1 single clone and/or plasmid within our hospital setting. Owing to a number of limitations, we could not exclude this possibility by determining plasmid profiles and pulsed-field gel electrophoresis patterns of the isolates. As the available treatment options are limited, antibiotic control policies together with the implementation of infection control measures remain of high importance.

Conclusion

The essential oil and the ethanolic extract of *C. zeylanicum* showed varying degrees of antimicrobial activity against the ESBL producing multidrug resistant isolates. The presence of phytochemicals may be responsible for its therapeutic effects. This plant could be a source of new antibiotic compounds which could be more effective against multidrug resistant strains of microorganisms. To test and identify the specific antimicrobial compounds, further work is needed.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The author expresses her thanks to Dr. Rehab Abdeen Herzallah Assis, Prof. Of chemistry at King Khalid University, for her helping in chemical analysis work.

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