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Novel Medical Properties of *Cinnamomum zeylanicum* Oil against *Pseudomonas aeruginosa*

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Cinnamon oil has variety of medical properties against several types of Gram-positive and Gram-negative bacteria, several recent studies deals with the common medical properties of cinnamon oil and its application on infection control and wound management. Methods: This study illustrates new medical properties of cinnamon oil against one of the most virulent pathogens which is an opportunistic organism; *P. aeruginosa* a Gram-negative pathogens with several virulence factors that we will deal with and how cinnamon oil can overcome its activity. In this study we focus on the virulence activity of *P.aeruginosa* and how it can be a main reason of chronic wounds infection, inflammation and necrosis. The clinical isolates under investigation in this study are pathogenic isolated from different wounds, ulcers and bed sores from patients of Alexandria university hospitals. These new medical properties of cinnamon oil can hinder the pathogenic properties of *P.aeruginosa* through facing its virulence factors and thus achieving of complete recovery.

Key words: Virulence factors, Proteolysis, Haemolysis, antibacterial, anti-proteolytic.

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that may be isolated from different habitats, it is characterized with high survival rate due to its high adaptability and ability to resist antibiotic activity, and it may cause severe infections as severe burns, diabetic foot infections, bed sores, eye infection and cystic fibrosis (CF). The treatment of *P. aeruginosa* becomes very limited due to the increase of antimicrobial agents resistance which leads to high rate of resistance (Lyczak *et al*. 2000).

The incomplete recovery of an acute infection with *P. aeruginosa* may lead to a chronic infection with an increase in biofilm formation (Williams *et al*. 2010). The isolation of *P. aeruginosa* from acute infections is different from those isolated from chronic infections in phenotype (Smith *et al*. 2006). The comparison between isolates which are isolated from acute infections that lack some of the virulence factors from those isolated from chronic infections, in other hands pathogens from chronic infections may lack some of the inflammatory virulence factors such as flagella and pili, but instead it has other virulence mechanisms as type 3 secretion system (T3SS) which contain different exotoxins (ExoU, ExoS, ExoT and ExoY) (Hogardt & Heesemann 2010).

P. aeruginosa isolates from chronic infections can form biofilms which are mucoidal because of the exopolysaccharide alginate (Sadikot *et al*. 2005; Kipnis *et al*. 2006). In biofilms bacteria are attached to each other in a highly organized structure to the surface of the infected tissue linked with quorum sensing (Bjarnsholt *et al*. 2010), this structure consists of extracellular polymeric substances (EPS) which are formed from polysaccharides, nucleic acids, lipids, and proteins

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that conform the resistance mechanism and prevent antibiotic activity (Hall-Stoodley L & Stoodley P 2009; Lieleg *et al*. 2011).

P. aeruginosa is able to secrete several proteases which cause have the main role in infection and sepsis and they are able to degrade immunoglobulins and fibrin, contribute to tissue damage and affect epithelial cell junctions (Kipnis *et al*. 2006).

Host proteins and host fibronectin are known to be degraded by alkaline protease which secretes zinc metallo-protease that is one of *P.aeruginosa* proteases (Laarman *et al*. 2012). The external cell wall of *P.aeruginosa* composed of lipopolysaccharides such as lipid A and O-polysaccharide which have roles in antigenicity as interactions with antibiotics, inflammatory response and exclusion of external molecules (King *et al*. 2009).

Another virulence factor which is secreted by *P. aeruginosa* and can contribute to its pathogenicity is exotoxin A, that inhibition of protein synthesis leading to cell death (Schultz *et al*. 2000). Chronic inflammation may be a direct cause of bacterial infection to a wound or ulcer, a lot of ways or treatment are used for overcoming inflammation but non-steroidal anti-inflammatory drugs (NSAIDs) are the most used pharmacotherapy buts its prolonged use may be very expensive beside it increase the risk of heart failure diseases and may lead to stroke or myocardial infarction (Rainsford 1999).

Herbal medicines are an important source for the discovery of new drugs, such as salicylic acid from *Salix alba* and have been used for inflammatory diseases treatment and its development is commonly known as aspirin, there are Many other medicinal plants were for their anti-inflammatory activity and for the development of new drugs (Ji *et al*. 2009). Inflammation is a biological process which is a response or injury of tissues, its clinical features are redness, warmth), swelling and pain, which are firstly described by Aurelius Cornelius, a Roman physician (Huang *et al*. 2011 , Yoon & Baek 2005).

Cinnamon is well known for its medical properties as it is used for stomach cramps (Braun 2006), intestinal spasms, nausea, and flatulence, to improve the appetite, and to treat diarrhea (Winston 2007), also has benefits in controlling of blood glucose levels in diabetes (Vera 2010), lowering the levels of the bad cholesterol LDL (low-density lipoprotein) (Qin Y *et al*. 2009), and for healing of wounds (Farahpour & Habibi 2012).

Cinnamomum zeylanicum has the ability to activate the immune system by regulating anti- and pro-inflammatory responses (Cao *et al*. 2008), *C. Zeylanicum* regulates gene expression involving inflammatory insulin, and lipoprotein signalling pathways (Qin B *et al*. 2009).

In this study we will focus on other properties of *Cinnamomum zeylanicum* oil which are not intensively studied, and how they compete the pathogenic properties of *P. aeruginosa.*

MATERIALS AND METHODS

Photogenic six *P.aeruginosa* isolates had been isolated from different wounds, ulcers and bed sores from the labs of Alexandria university hospitals, pure cinnamon oil (Sigma, Egypt), Photometric diagnostic devise (Star dust V4, Diasys, Germany), nutrient agar and MacConkey agar (Oxoid, England).

Isolation of pathogens

The bacterial samples had been collected deeply from infected tissues using sterile swabs and inoculated immediately into blood agar plates and MacConkey plates and incubated for 24 hours at 37°C.

General characterization of *P. aeruginosa*

The general characteristics of P.aeruginosa has been simply detected morphologically, as the detection of the green pigment of pyocyanin, the hemolytic activity of the bacterial growth on blood agar plate and the proteolytic activity of the pathogen on the casein plate. Blood agar plates can be used for detection of the hemolytic activity of the isolates, as the organism can affect the membrane of the erythrocytes causing haemolysis. Skimmed milk can be added to nutrient agar to produce casein agar plate with high protein content which used for detection the ability of pathogen on protein degradation (i.e.; proteolysis).

Detection of proteolytic activity of *P. aeruginosa*

The proteolytic activity of P.aeruginosa originates as known from the proteins and enzymes that were secreted by the cell wall of the organism, so to detect the anti-proteolytic and also hemolytic activity we have no need to use the whole cell

of the pathogens but we just need to use its free cell extract. The free cell extracts were detected centrifugation of broth which had been inoculated with the pathogen and incubated at 37°C over night, for 10 minutes at 4500 rph. The cell free extracts were tested for their proteolytic activity through disk diffusion methods on a casein plate and the activity was detected by detection of the zone of proteolysis (Casein degradation).

Detection of hemolytic activity of *P. aeruginosa*

Haemolysis is the ability of the organism to degrade the erythrocytes, and this test was done on blood agar plate. The test was done by using the free cell extract and by using the disc diffusion method and the results were determined through detection of the haemolysis zone.

General characteristics of cinnamon oil

The general characteristics of cinnamon oil was tested against the pathogens on different media plates with disc diffusion method, nutrient agar for antibacterial, blood agar for non hemolytic property and casein plate for anti-proteolytic activity.

Antibacterial activity of cinnamon oil against *P. aeruginosa*

The antibacterial activity of cinnamon oil was tested against all the clinical isolates on sensitivity method on agar plate and the antibacterial activity was tested with inhibition zone.

Anti hemolytic activity of cinnamon oil against hemolytic *P. aeruginosa*

The anti-hemolytic activity of the cell free extracts of the pathogenic organisms was detected through photometric method.

This experiment was done by 13 EDTA blood tube, six tube was inoculated with 10µl of the

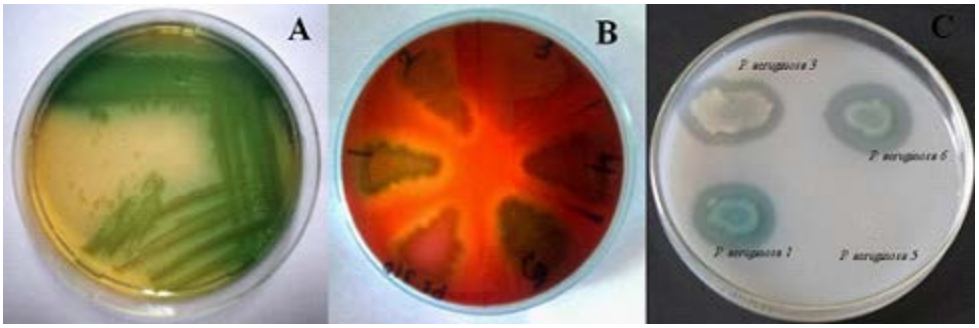

Fig.1. Characteristics of *P. aeruginosa*,(A)Green *P. aeruginosa*1,(B) Hemolytic activity of the six *P. aeruginosa* bacterial isolates,(C) The proteolytic activity of *P. aeruginosa* 1,3,5,6

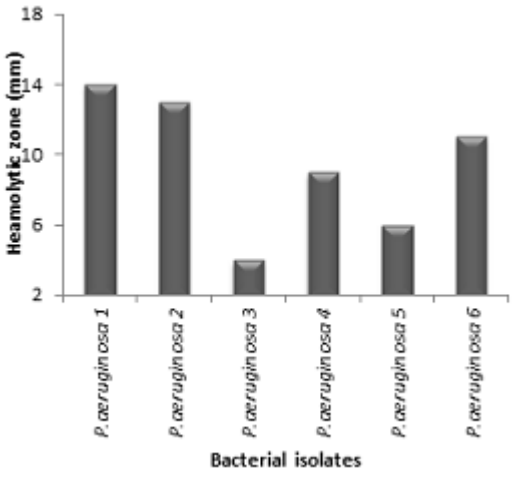

Fig. 2. Hemolytic activity of the six *P.aeruginosa* isolates

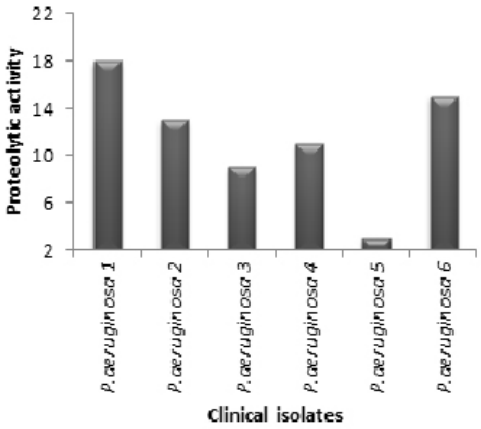

Fig. 3. Proteolytic activity of *P.aeruginosa*.

cell free extracts of the six pathogenic organisms, the other six tubes was inoculated with 10µl cell free extract and 10µl of cinnamon oil and the thirteenth tube was a control EDTA blood, the thirteen tube was incubated for 24 hour and then centrifugated and the plasma tubes were tested at wave length (340 nm).

Anti- proteolytic activity of cinnamon oil against proteolytic *P. aeruginosa*

This experiment was done by 13 casein broth tubes, six tubes were inoculated with 10µl of the six pathogen cell free extracts, another six tubes were inoculated with 10µl of the bacteria cell free extracts and 10µl of cinnamon oil and the thirteenth tube was a casein broth control, the thirteen tube was incubated for 24 hour and then centrifugated and the supernatant absorbance was measured at wave length (340nm).

RESULTS

Microbial properties of *P. aeruginosa*

The figure (Fig.1) illustrated that (A) the normal growth of *P. aeruginosa* 1 on nutrient agar, which shows the green pigment of pyocyanin; one of *P. aeruginosa* virulence factors (B) the hemolytic activity of the six bacterial isolates shows that the six isolates have hemolytic activity on blood agar and (C) the proteolytic activity of the bacterial isolates *P. aeruginosa* 1,3,5,6 on casein plate showing the degradation of casein on the plate with proteolysis enzymes secreted by *P. aeruginosa*.

Hemolytic activity of P.aeruginosa

The following (Fig.2) showing the hemolytic activity of the clinical isolates of *P. aeruginosa* and illustrated that *P. aeruginosa* 1 has

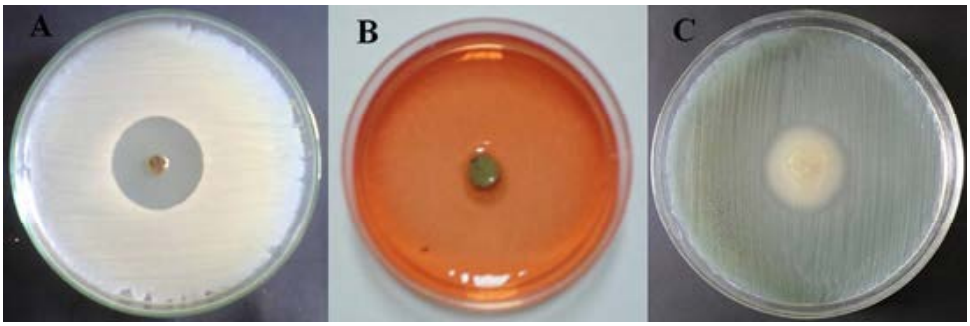

Fig. 4. Characteristics of *Cinnamomum zeylanicum* oil,(A)Antibacterial activity aginst *P. aeruginosa2*,(B) The Non-Hemolytic activity *Cinnamomum zeylanicum* oil,(C) The anti-proteolytic activity of *Cinnamomum zeylanicum* oil against *P. aeruginosa* 1

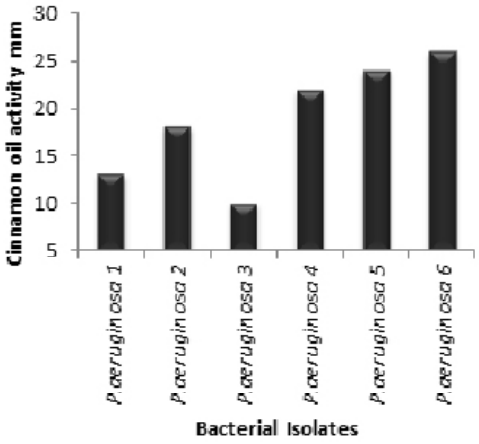

Fig. 5. Antibacterial activity of *Cinnamomum zeylanicum* oil against the six *P.aeruginosa* isolates

the highest hemolytic activity (14 mm IZ) while *P. aeruginosa* 3 has the lowest hemolytic activity (4 mm) and the other isolates have a variation in hemolytic activity as *P. aeruginosa* 2 has hemolytic activity of (13 mm), *P. aeruginosa* 4 (9 mm), *P. aeruginosa* 5 (6 mm), P. *aeruginosa* 6 (11 mm).

***Proteolytic activity of* P.aeruginosa**

The proteolytic activity of the *P.aeruginosa* clinical isolates was illustrated through (Fig. 3), the results showed that *P.aeruginosa* 1 was the most proteolytic isolate (18 mm IZ), while *P.aeruginosa* 5 was the lowest proteolytic isolate (3 mm) and the other isolates had a variety proteolytic activity as *P. aeruginosa* 2 (13 mm), *P. aeruginosa* 3 (9 mm), *P. aeruginosa* 4 (11 mm), *P. aeruginosa* 6 (15 mm).

Characteristics of *Cinnamomum zeylanicum* oil
General Characteristics of Cinnamomum zeylanicum oil against P.aeruginosa.

The following figure (Fig. 4) illustrated some of the intensive studied properties of *Cinnamomum zeylanicum* oil and this properties were tested against *P.aeruginosa* isolates which are under investigation during this study as (a) cinnamon oil has a potent antibacterial activity against *P. aeruginosa* clinical isolates, (b) cinnamon oil has no hemolytic activity and (c) cinnamon oil has anti proteolytic activity against *P.aeruginosa* clinical isolates which already has proteolytic activity.

Detection of cinnamon oil activity

The antibacterial activity of cinnamon oil is discussed in the following (Fig. 5), the results show that cinnamon oil has antibacterial activity against all the pseudomonal clinical isolates, *P.aeruginosa* 6 has the highest level of antibacterial activity (26 mm IZ), and the *P.aeruginosa* 3 have the lowest activity (10 mm), while *P.aeruginosa* 1 (13 mm), *P.aeruginosa* 2 (18 mm), *P.aeruginosa* 4 (22 mm) and *P.aeruginosa* 5 has antibacterial activity (24 mm).

Anti-Hemolytic activity of *Cinnamomum zeylanicum* oil against *P. aeruginosa*

The haemolysis of blood is measured through photometric devices (Star dust V4, Diasys, Germany) at wave length (340 nm) and the results illustrated in (Table 1) showed the haemolysis of blood plasma results from inoculation with bacterial cell free extract and the anti-hemolytic activity of cinnamon oil in inoculated blood tube

Table 1. Anti-Hemolytic activity of Cinnamomum zeylanicum oil against P.aeruginosa

Bacterial Isolates	Plasma Control	Determination of protein content	
		Reading of blood plasma inoculated with bacterial cell free extract	Blood plasma inoculated with bacteria cell free extract and treated with *Cinnamomum zeylanicum* oil
P.aeruginosa 1	0.1	1.85	0.2
P.aeruginosa 2		1.72	0.15
P.aeruginosa 3		0.7	0.19
P.aeruginosa 4		1.1	0.14
P.aeruginosa 5		0.9	0.1
P.aeruginosa 6		1.5	0.12

Photometric reading result of the *Cinnamomum zeylanicum* oil alone = 0.1±0.1

Table 2. The anti-Proteolytic activity of *Cinnamomum zeylanicum* oil for the six isolates of *P.aeruginosa*

Bacterial Isolates	Control casein broth	Determination of protein content	
		Casein broth inoculated with bacterial cell free extract	Casein broth inoculated with bacterial cell free extract and treated with *Cinnamomum zeylanicum* oil
P.aeruginosa 1	8.2	0.2	8.4
P.aeruginosa 2		0.4	8.3
P.aeruginosa 3		0.5	8.6
P.aeruginosa 4		0.6	8.6
P.aeruginosa 5		1.2	8.7
P.aeruginosa 6		0.1	8.4

Photometric reading result of the *Cinnamomum zeylanicum* oil alone = 0.4±0.2

and the results were compared with blood plasma. The results illustrate that the absorbance reading of plasma control is (0.1), while the absorbance readings of the hemolytic plasma resulting from the inoculation with bacterial cell free extracts range from 0.7 to 1.85 and the readings of plasma treated with cinnamon range from 0.1 to 0.2.

The anti-Proteolytic activity of *Cinnamomum zeylanicum* oil against *P. aeruginosa*

The anti-Proteolytic activity of *Cinnamomum zeylanicum* oil for all the six isolates of *P.aeruginosa* was determined as all the protein content of the casein broth (control) were 8.2 mg/dl, while the casein broth inoculated with bacteria contain a protein content ranges from (0.1 mg/dl) to (1.2 mg/dl) and the casein broth inoculated with bacteria and cinnamon oil contain protein content ranges (8.3:8.7 mg/dl ±0.2). *Cinnamomum zeylanicum* has anti-proteolytic activity illustrated in (Table 2).

DISCUSSION

Recently a lot of studies discuss the pseudomonal infection, the pathogenecity, epidemiology and methods of treatment. In this study we have contrast to some of the virulence factors that affect the treatment of pseudomoal infections and how cinnamon oil has a stand towards them.

The results showed that *P. aeruginosa* is a Gram-negative pathogen characterized with unique green color due to presence of pyocyanin pigment which is one of the most known virulence factors of *P.aeruginosa*, this agrees with that pyocyanin is highly toxic for human epithelial surfaces, may cause adenosine mono phosphate fall and activate proteolysis by inactivation of α1-protease inhibitor (Ran *et al*. 2003). The results show that all the clinical isolates of *P.aeruginosa* have hemolytic activity due to the presence of *P.aeruginosa* exotoxins that are able to attack the membranes of the red blood cells causing their rupture which is a critical role of virulence (Buchon *et al*. 2009; Jiang *et al*. 2009; Vodovar *et al*. 2005).

Beside the activity of pyocyanin and the hemolytic activity of *P. aeruginosa* the results illustrate that *P. aeruginosa* has another virulent activity which is the most potent and active one, the results show that all the isolates exhibit proteolytic activity which can affect the treatment of the infected tissue through delaying of healing by proteases enzymes which can lead to necrosis or fibrosis of the infected tissue as Proteases are enzymes secreted by *P. aeruginosa* which are able to break the peptide bonds between the peptide chains in protean structure (Barrett *et al*. 2004), so pathogenic *P. aeruginosa* proteases can interact with their hosts cells causing protein digestion.

Cinnamon oil is recently studied for its application on medical branch of research as it has a lot of benefit properties; in this study we discuss the antibacterial activity of cinnamon oil as the results show that cinnamon oil has a potent antibacterial activity against all the clinical isolates of *P.aeruginosa* which agrees with that of cinnamon oil shows inhibition to different Gram Positive and Gram Negative pathogenic bacteria (Lopez *et al*. 2005).

To determine the complete recovery the treatment must obtain the properties that make it able to be effective against the infection causing organism antibacterial, anti-hemolytic, anti-proteolytic and anti-biofilm. Our results show that cinnamon oil has antibacterial activity against the pathogenic organism with non hemolytic activity against the host blood; it also has anti hemolytic activity against the hemolytic *P. aeruginosa* pathogens and cinnamon oil has anti-proteolytic activity against proteolytic active *P. aeruginosa*, these properties enhance the ability of cinnamon oil to achieve the full recovery of the infected tissue, also cinnamon oil has anti-inflammatory effect which decrease inflammation during treatment (Liao *et al*. 2012).

CONCLUSION

Cinnamon oil properties such as antibacterial, anti-hemolytic, anti-proteolytic and anti- inflammatory can hinder the microbial properties of pathogenic *P.aeruginosa* and virulence factors such as proteolytic and hemolytic activity.

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