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Validity of Chitosan-Cinnamon Formula for Treatment of MDR-Pseudomonal Wound Infections

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The growing threat of Multi-resistant (MDR) *P. aeruginosa* had been widely distributed through infection of chronic wounds, urinary tract, and diabetic feet and also nosocomial infection of hospitals. This study was planned to solve the problem of the resistance of MDR *P. aeruginosa* to most recent antibiotic in pseudomonal infected patients, and to produce a new treatment of natural source which has anti bacterial activity and wound healing effect with no side effect as chitosan is a natural polymer used widely in wound management with its unique non toxic nature. Cinnamon oil has a variety of medical a applications as anti-microbial, anti -ulcers and anti- inflammatory effect. Our study included 100 patient admitted to Alexandria University Hospital with different wound and ulcers infections. Total of 127 pathogens were isolated, 43 (30%) were *P. aeruginosa*. Most resistant six *P. aeruginosa* isolates were chosen for separated study. Chitosan and cinnamon oil were prepared from their natural sources. The results ended with a final formula of some natural component which has anti-bacterial activity, non-inflammatory, wound healing activity, with no toxic side effects. It could be included that the recent intensive used antibiotics can be replaced with the prepared natural formula as it can help in the full recovery of the wounds faster and safer than antibiotics.

Key words: MDR- Pseudomonal wound infection, Nosocomial infection, Natural polymers, Chitosan, Cinnamon oil.

P. aeruginosa is a pathogenic rod – shaped Gram negative bacteria which can be isolated from several infected tissues, it is an opportunistic organism which only infect immune-compromised patients. It can survive on moisture corners, walls and floors of hospitals[1].

P. aeruginosa is the main cause of several sever infectious diseases such as UTI (Urinary tract infection), CF (Cystic fibrosis), and also wound and burn infection[2]. To define a pathogenic organism as MDR- Organism, it must be resistant to one of the antibacterial agents of (carbapenems, fluroquinolones and aninoglycosides)[3].

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The threat of MDR- *P. aeruginosa* had been increased and become intensively studied by the scientists everywhere as *P. aeruginosa* acquired resistance against most of the recent used antibacterial agents(Antibiotics)[4].

P. aeruginosa enhance its resistance through several mechanisms such as efflux pumps, or it can form biofilms, it can produce several enzymes which van inhibit the activity of some antimicrobial agents like ²-lactam it produce ²-lactamase and aminoglycosides it produce aminoglycoside modified enzymes[5].

Cinnamum zeylanicum is popularly known in Egypt as cinnamon. Cinnamon is obtained from dried barks or leaves of an ever green trees which are growing in very high temperature regions, it was been widely used in food cooking due to its special flavor and its

aromatic properties, and also it was introduced it a lot of industries in addition to its antifungal[6], antibacterial[7], pesticidal properties.

Cinnamon oil, as it used in food production and cooking has no harmful side effects to human, while it inhibit the growth of molds, yeast, fungi and food poisoning bacteria so it can be used as food preservative, as it is characterized by hydrophobic properties so it can bind to the cell membrane lipid bi-layer affecting respiration and production of energy[8], which lead to leakage of some cell contents[9,10] it also can inhibit the bacterial enzymes system inhibiting the bacterial mode of action[11].

Chitosan is the second most common polysaccharide on earth, which can be used widely in wound care and treatment due to its haemostatic properties, it is also characterized by many biological properties as it help in wound healing and stimulate proliferation of tissues and it has antibacterial, antifungal and wound treatment[12-19].

Chitosan is a non –toxic biodegradable polymer which has several bio active properties against wide variety of organisms it is characterized by antifungal and antibacterial properties which are helpful properties in biomedical skin care and other application like water purification, Chitosan gained its properties from the chemical reaction of protonation of NH_2–groups on chitosan molecule structure [17].

Chitosan has its special mode of action in wound healing mechanism, it helps in fasting of wound healing through mechanism of depolymerize to release N-acetyl-b-D-glucosamine , this helps in collagen deposition in wound site and this helps blood clotting naturally[12,17] .

MATERIALS AND METHODS

Bacterial samples were collected from patients on sterile swabs. All used antibiotic discs from (Oxoid company, England), chitosan was prepared 89% deacytelation degree from three sources. Essential oils were prepared by steam distillation of specific part of each plant.

Collection of samples

Through swabbing from the infected wound, pus swabs were swabbed from the deepest part of the wound using sterile swabs and transported to the microbiology laboratory (within 4 hrs maximum). They were cultured on blood agar plates, MacConkey medium and nutrient agar. The media were incubated at 37°C overnight aerobically and the isolated bacteria were identified by biochemical identification methods.

Extraction of essential oils

Essential oils were prepared by steam distillation from different parts of eight different herbs through hydro distillation. Dried parts of the eight herbs were purchased from local retail markets, then were grounded using a grinder into a fine powder, then they were kept in dark bottles. Finely ground herb (35g) was hydro distillated in 375 ml of DW. Then essential oils were collected and extracted from water using n-hexane in separation funnel. Hexane fractions were poured into a rotary evaporator flask and concentrated by vacuum evaporator until all of the hexane was completely evaporated, leaving the absolute oils[20].

Chitosan preparation

Chitosan was prepared from 3 different sources of crustaceans shells and dissolved in 2% acetic acid by concentration of 1% and deacetylation degree of 89%, through the steps illustrated as following.

Shell wastes from food processing (Crab, Shrimp, Squilla) were collected, and chitosan preparation was done through three main steps (a) decalcification in HCL 50% for 1 hour, (b) deproteinization over night in NaoH (3:1) which lead to formation of chitin, (c) deacetylation with NaoH overnight and wash with distilled water and dissolve with 2% acetic acid[21,22].

Testing antibacterial activity of antibiotics, cinnamon and chitosan

By using Kirby–Bauer disc diffusion method using commercially purchased antibiotic discs and interpreted according to CLSI 2007[23]. All plates were incubated at 37°C and results were taken after 24 hrs. Determination of the used cinnamon oils and chitosan concentration is by simple dilution methods to have the lowest concentration with have an acceptable antibacterial activity.

In vivo evaluation of the final formula

The evaluation of the final formula done on experimental rats, through two different steps:

The morphological evaluation

By evaluation the morphology of infected

wounds done on backs of six experimental rats and the healing of these wounds.

The hematological evaluation

By evaluation of the hematological differences in the rats blood films through three different stages of treatment (Before, During and After treatment), the blood of rates tested using an automatic hematological device Diatron Hematology-Analyzer (Abacus 5).

RESULTS

Detection of antibiotic sensitivity profile

The 6 isolates were tested against 16 of the most common antibiotic according to CLSI 2007 (23) (Figure 1).

In this study it was recorded that imipenem had the most antibacterial activity among all the antibiotics with average of activity (26.8 IZ mm) and vancomycin had the lowest antibacterial activity (1.8 mm), while doxacyclin, linezolid, erythromycin, AMP/SUL, methicillin, and ceftazidime had no antibacterial activity on all the bacterial isolates.

Antibacterial activity of Essential oils

Essential oils were tested against six pseudomonal isolates, to detect which oil has the greatest antibacterial activity (Figure 2).

The results showed that oil of *Cinnamomum zeylanicum* (Cinnamon oil) had the highest antibacterial activity of average (18.83 IZ mm) and the oil of *Allium cepa* had the lowest antibacterial activity of average (1.17 mm), while the oils of *Mentha longifolia* and *Ocimum basilicum* had no antibacterial activity against pseudomonal isolates.

Detection of chitosan antibacterial activity

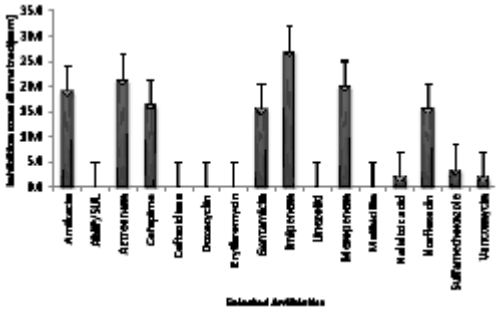
Three different types of chitosan which prepared from different crustaceans were tested against the six Pseudomonal isolates (Figure 3).

It was recorded that the chitosan prepared from the *Squilla mantis* (SqC) had the highest antibacterial activity with an average of 16.0 IZ mm and the shrimp chitosan had average activity of 13.8 mm while crab chitosan had the lowest average antibacterial activity (11.2 mm).

Detection of cinnamon-chitosan synergism

Cinnamon oil showed antibacterial activity with an average of 10.0 IZ mm and squilla chitosan showed average antibacterial activity of 15.0 mm while the combination of both cinnamon oil and squilla chitosan showed average antibacterial activity of 35.0 mm against *Pseudomonas* 3 (Figure 4) (a).

Among the six isolates under investigation the usage of antibiotic gave an antibacterial activity of the highest average (26.83 IZ mm for imipenem), cinnamon oil gave an average activity of 26.0 mm and also squilla



AMP/SUL= Ampicillin & Sulpactam

Fig. 1. The Antibiotic sensitivity profile of the six Pseudomonal isolates.

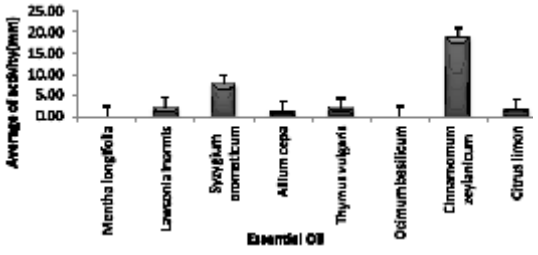


Fig. 2. The average of antibacterial activity of 8 different essential oils (EO) against the 6 pseudomonal isolates

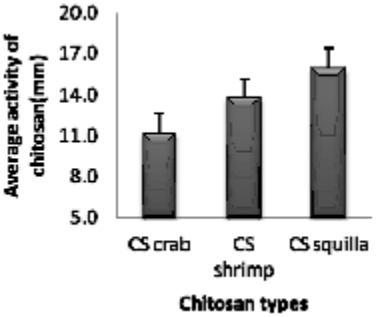


Fig. 3. The antibacterial activity of three different chitosan types.

chitosan gave an average of activity 20.0 mm while the synergistic combination of chitosan and cinnamon oil gave an average of activity ranged from 35.0 to 53.0 mm(Figure 4) (b).

Determination of cinnamon-chitosan dilution

The final formula was chosen according to lowest active concentration and toxicity profile will be illustrated below.

The chosen concentration of both cinnamon oil and chitosan was determined by (Table1) as the used concentration is the lowest concentration which has antibacterial activity, in case of cinnamon oil the used concentration is 150¼l/mL, and chitosan with concentration of 200¼g/ml.

Detection of Cinnamon oil and squilla Chitosan toxicity

Biotoxicity test was done by adding (100

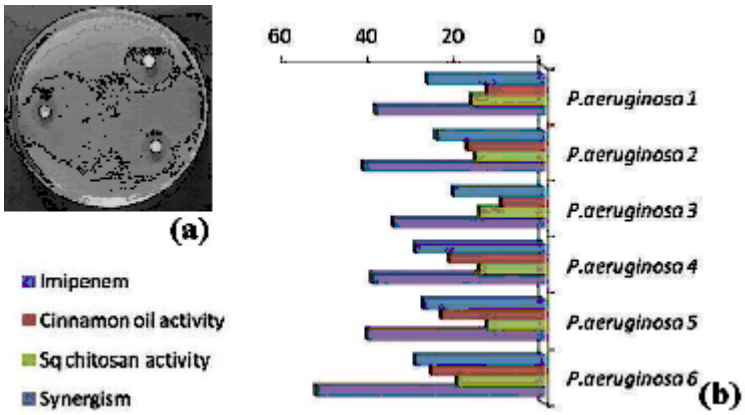


Fig. 4. (a) Disk diffusion method for determination of synergism between cinnamon oil (Cin) and squilla chitosan (SqC). (b) Competitive chart showing the difference of antibacterial activity between each of cinnamon oil, squilla chitosan, their synergistic formula and antibiotics.

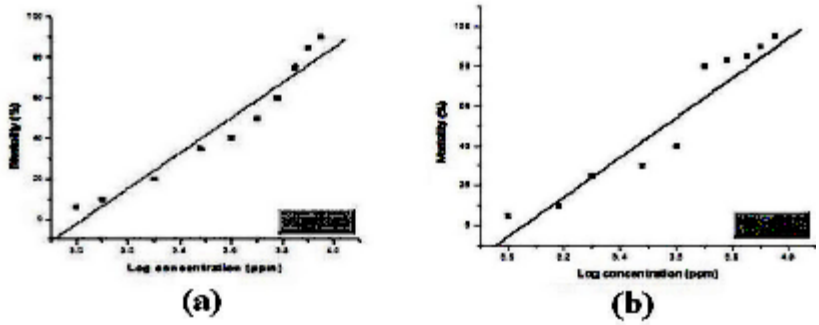


Fig. 5. Determination of the LC_{50} from the toxicity profile of **(a)** squilla chitosan**, (b)** Cinnamon oil

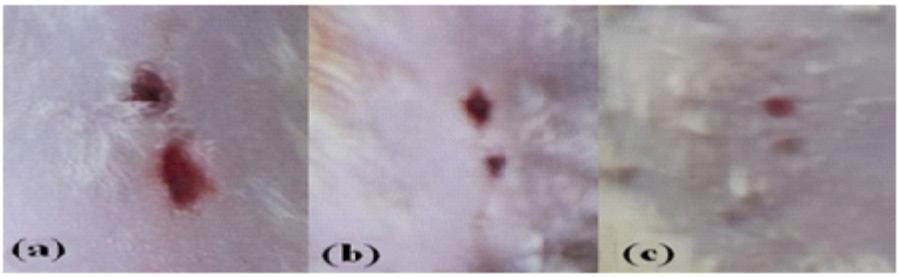


Fig. 6. The treatment stages of an infected wound with the formula, (a) Before treatment, (b) During treatment, (c) After treatment

Table 1. Determination of antibacterial activity with different dilutions

Cinnamon oil dilutions with in DMSO (W/W)%	Inhibition zone mm						Chitosan dilutions with in Acetic acid 2% (V/V)%	Inhibition zone mm					
	P. aeruginosa 1	*P. aeruginosa 2*	*P. aeruginosa 3*	*P. aeruginosa 4*	*P. aeruginosa 5*	*P. aeruginosa 6*		*P. aeruginosa 1*	*P. aeruginosa 2*	*P. aeruginosa 3*	*P. aeruginosa 4*	*P. aeruginosa 5*	*P. aeruginosa 6*
100	22	23	24	21	23	24	100	17	16	15	15	13	20
90	19	22	22	20	20	21	90	15	15	14	13	12	19
80	16	21	21	19	17	19	80	12	14	13	12	11	17
70	14	20	18	16	15	16	70	11	12	12	11	10	16
60	12	18	17	13	14	14	60	10	11	10	10	9	14
50	11	14	13	12	13	11	50	9	10	9	9	8	11
40	10	10	10	9	11	9	40	7	8	8	8	7	9
30	8	9	8	8	10	8	30	6	7	5	6	5	8
20	7	7	6	5	8	6	20	5	4	4	5	4	6
15	4	3	2	4	3	4	15	0	0	0	0	0	0
10	0	0	0	0	0	0	10	0	0	0	0	0	0
DMSO	0	0	0	0	0	0	Acetic acid 2%	0	0	0	0	0	0

DMSO: Dimethyl sulfoxide

live *Artemia salina* nauplii) in 3 cm diameter containing 2 ml seawater. 10 µl from each of squilla chitosan and cinnamon oil which are under investigation were added in each plate and observed after 24 hrs (24).

Toxicity of Chitosan

From the illustrated (Figure 5), (a) The LC_{50} for squilla chitosan was (3.565) which was a concentration of (3672.8 µg/ml) and was so much more than the used concentration.

Toxicity of Cinnamon oil

From the illustrated (Figure 5), (b) The LC_{50} for cinnamon oil was (3.51) which was a concentration of (3235 µl/ml) and was so much more than the used concentration.

The previous figure showed that (a) a superficial wound had been done in the back skin of an experimental rat which had been infected by *P. aeruginosa* 6 (most resistant isolate) with bacterial dose of 10^5 CFU. (b) Showed an intermediate phase of treatment with semi-treated features. (c) Showed the final phase of complete treatment and healing after exposing to regular doses of the formula.

Hematological Evaluation

With the bacterial infection an increase in the total WBCs count had occurred and an increase may occur in the differential count of neutrophilis or lymphocytes (Table 2).

The previous results (Table 2) showed the hematological evaluation of the final formula on the experimental rats blood (a) Before treatment, there was an increase in the total WBCs Count (19.3 x 10^3c/mm^3) with an increase in neutrophils count (88%), (b) During treatment, the formula could combat the infection with an observed decrease in the total WBCs count (13.2 x 10^3c/mm^3) and the neutrophils count was (79.6%) (c) After treatment, at the complete treatment and healing phase, the total WBCs count returned to its normal range (8.9 x 10^3c/mm^3) and also the neutrophils (61.3%) which was a clear evidence for complete recovery.

DISCUSSION

This study describes the usage of some natural substances and material which can be used in treatment of MRD- Pseudomonal infected

Table 2. Hematological analysis for experimental rats blood through 3 different stages of treatment using Diatron Hematology-Analyzer (Abacus 5).

Total W.B.Cs	Before treatment 19.3 x 10^3c/mm^3	During treatment 13.2 x 10^3c/mm^3	After treatment 8.9 x 10^3c/mm^3	REF.Range 3.8 - 10.0
Differential Count				
LYM%	5.8	13.3	29.2	20 – 40
MON%	3.2	4.5	6.3	2 – 8
NEU%	88	79.6	61.3	55 – 70
EOS%	3	2.6	3.2	1 – 4
BAS%	0	0	0	0.5 – 1

wounds. In this study the isolation of six MDR-*Pseudomonas* isolates was done and tested against 16 of the most common used antibiotics in Egypt which resulted in (imipenem, aztreonam, meropenem, amikacin, cefepime, norfloxacin, gentamicin, sulfamethoxazole, nalidixic acid and vancomycin) have average of antibacterial activity against all isolates ranges (26.8 mm – 1.8mm),while (ceftazidime, methicillin, AMP/SUL, erythromycin, linezolid, doxacyclin) has no antibacterial activity against any of isolates.

This results confirm those of previous study of some antibiotics which are active against *Pseudomonas* such as (cefoperazone, ceftazidime, cefepime, aztreonam, imipenem and meropenem), aminoglycosides (gentamicin and amikacin) and fluoroquinolones[25, 26], but it disagree with the same study in the case of ceftazidime and some ²-Lactams (methicillin and AMP/SUL).

The current study deal with the usage of some herbal essential oils and this agree with the study of[27] and others that a lot of studies deals with essential oils and some herbs for many years as food preservative against food borne bacteria and for herbal medicine[28, 29].

Essential oils are considered as a natural source of antimicrobial compounds[30] which used for treatment of some pathogenic infections. In vitro studies in this work showed the antibacterial activity of some essential oils against pathogenic MDR- *Pseudomonas* isolates. The antibacterial activity had been tested and the results show that cinnamon, clove, henna, thyme, lemon, onion have antibacterial activity against *pseudomonas* isolates. Several studies[7, 31] have shown that cinnamon and clove oils had strong and consistent inhibitory effects against various pathogens.

In this study the results shows that the three types of chitosan prepared from three different sources crustaceans shells (shrimp,Crab,Squilla) has antibacterial activity range of (11.2 – 16.0 mm) and this agrees with the study of Chitosan is a non-toxic bio active polymer which has antibacterial activity against gram positive and gram negative[32, 33]. In the evaluation section the results shows how the formula and its components helps in wound treatment through two strategies, the first one that the formula has antibacterial activity because of cinnamon oil and chitosan, and the chitosan which is a basic component in the formula play an important role in wound healing which agree with the previous study that Chitosan can help in fasting wound healing and produce smooth tissues over the wound site through collagen fibril production enhancing[34,35].

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