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xEvaluation of Anti-adherent Activity of Excretions of Irradiated *Lucilia sericata* Maggot and Certain Essential Oils against Some Pathogenic Bacterial Strains

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ESSENTIAL OILS are widely used for their medicinal properties. They block adhesion and colonization of pathogenic microbes to epithelial cells which associated with bacterial resistance to antibiotics. So, this study investigates the effect of *Lucilia sericata* (flesh fly-an ectoparasite) excretions of non-irradiated and irradiated maggot and some essential oils on biofilm formation by tube method, antimicrobial susceptibility by agar disc diffusion method as well as on their anti-adherent activity by spectrophotometric method. The results showed that excretions and secretions (E/S) of non-irradiated and irradiated maggots (at 20 Gy), as well as (clove and cinnamon oils) did not have antibacterial activity against the tested bacterial strains *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*St. aureus*) and *Staphylococcus epidermidis* (*St. epidermidis*) except marjoram oil which has low antimicrobial activity against all the tested strains. The results also showed that the most potent oil was clove which decrease biofilm of *P. aeruginosa* by 83%, followed by marjoram (69%), then E/S of non-irradiated maggots (66%). Whiles, biofilm was less affected by cinnamon oil and E/S of irradiated maggots by 50 % and 36%, respectively. In addition, clove oil and E/S of non-irradiated maggots affect the pre-adhered biofilm of *P. aeruginosa* by 57 and 45 %, respectively.

Conclusion: Clove oil flowed by marjoram had anti-adherent effect on *P. aeruginosa*. Greater inhibition of adhesion was observed by excretions of non-irradiated *lucilia sericata*.

Keywords: *Lucillia sericata*, maggot E/S, essential oils, pathogenic bacteria, anti-adhesive activity, γ-rays.

The use of synthetic materials for temporary or permanent implantation *i.e.*, central venous catheters, urinary catheters, have been accompanied by the emergence of implant-associated infection. Bacterial infections following

colonization and biofilm formation on these prosthetic materials represent the principal cause of morbidity in patients undergoing prosthetic implantation (Bonaventura *et al.,* 2004).*P. aeruginosa* is the most common gram negative bacteri found in nosocomial life threaten infections of immuno-compromised patients. It is responsible for high rates of morbidity and mortality. The success of this organism is largely due to the production of myriad of virulence factors including its ability to form intractable biofilm (Adonizio *et al.,* 2008). *St. aureus* is equally important because it is a major cause of antibiotic resistance infections in hospitals (Jaklic *et al.,* 2008). *St. aureus* became resistant to penicillin soon after its widespread release, and after a second drug, was used to control penicillin-resistant bacteria, the strains evolved that were also resistant to the second drug methicillin resistant *St. aureus* (MRSA). There have even been strains discovered that resistant to a third drug; vancomycin (Nigam *et al.,* 2006). *St.epidermidis* is one of the leading pathogens of nosocomial infections, particularly associated with foreign body infections. The organisms produces a glycocalyx "slime" that acts as a glue adhering to the surfaces of indwelling medical devices during device insertion and form biofilm (Otto, 2009). The failure of existing antibiotics to control infections makes it crucial to search for alternatives to currently available antibiotics. Natural occurring and synthetic compounds that interfere with microbial virulence factors thought to provide new treatment strategies (Adonizio *et al.,* 2006).

The larvae of green-bottle fly *Lucilia sericata* (ectoparasite) are the most commonly used for wound management, which are known to be able to infest living hosts and parasitize host tissue. Maggots of *Lucilia sericata* have helped save lives efficiently by effectively cleaning wounds and healing wounds including ones that might otherwise have been fatal. It is uncertain to what degree excretions and secretions of the maggots E/S have any effect on bacterial growth, which or how useful any bacteriostatic effects might be, since different studies have produced contradictory results (Cazander *et al.,* 2009[a]) and some species of bacteria may be naturally resistant to E/S (Jaklic *et al.,* 2008). Therefore, we suspected E/S could interfere with biofilm formation of the pathogenic strains. Low doses of Gamma irradiation were used to affect the fertility of the fly. In this paper, we will study the effect of 20 Gy on the antimicrobial and anti-adherent activity of maggot excretions after irradiation of the larvae.

Maggots were approved for therapeutic purpose by the US Food and Drug Administration in 2004 and maggot therapy was being used in around 1.000 medical centres in Europe and over 800 medical centres in the United State for the treatment of chronic wounds such as leg ulcers, pressure sours, diabetic necrotic ulcers, as well as infected surgical wounds, burns and trauma injuries (Nigam *et al.,* 2006). Also, another study has shown that essential oils of medicinal plants contain bioactive compounds such as carotenoids, flavonoids, pectin, vitamins and coumarins, which have shown antibacterial, antifungal and anti-adherent activity (Choi *et al.,* 2009).

In the study we have taken a step further by exploring the antibacterial and anti-adherent activities of E/S of non-irradiated and irradiated maggot, and essential oils of some medicinal plants on *P. aeruginosa, St. aureus* and *St. epidermidis.* These medicinal plants were chosen on the basis of their traditional use against infections caused or complicated by these selected pathogenic strains.

Materials and Methods

Bacterial strains

P. aeruginosa, St. aureus and *St. epidermidis* used in this investigation were supplied by the Drug Microbiology Laboratory, Drug Radiation Research Department, NCRRT. These bacterial isolates were cultivated on Nutrient agar, NA (Oxoid).

Irradiation facility

The irradiation of maggots was performed using Gamma Cell 40 with radioactive source cesium-137 at the NCRRT, Cairo, Egypt. The dose rate was 0.45 Gy/ min. Maggots were exposed to 20 Gy as a single dose, which biologically equivalent to a sterility dose of the fly (Spradbery *et al.,* 1983).

Preparation of maggots excretions and secretions E/S

The excretions and secretions of the maggots E/S was carried as described by Arora *et al.,* (2010), where *Lucilia sericata* were reared on raw meat in plexi-glass cages under controlled humidity and temperature conditions (25°C). The eggs were deposited on fresh meat and allowed to hatch to maggots. Instar 1 maggots were used after 24 h when they measured 2 mm in length. Maggots were irradiated at 20 Gy then non-irradiated and irradiated maggots were

transferred to a 15 ml sterile tube with phosphate buffer sulphate, to a density of 100 larvae per 200 µl of the buffer. They were incubated in the dark at room temp. (25°C) for 1 h. Resultant maggots E/S were transferred to another tube using a pipette then autoclaved for 20 min at 121°C. Subsequently, the E/S were allowed to cool to room temperature then, stored at -20°C for further use.

Source of essential oils used

Clove, cinnamon and marjoram essential oils of pharmaceutical grade were obtained from the local market, in Great Cairo.

Antimicrobial activity

Antimicrobial activity was carried out by the disc diffusion method described by Salie *et al.* (1996). The experiment was performed two times under strict aseptic conditions. Antimicrobial activity was determined by measuring the diameter of inhibition zone (mm) and the mean values were calculated.

Detection of slime production

A qualitative assessment of slime production for the tested bacterial strains was determined according to the technique described by Christensen (1982) with some modification by Farrag (2001). A loop of the tested organism from a nutrient agar plate was inoculated into a glass tube containing 5 ml of trypticase soy broth (TSB, England) containing glucose 0.25% w/v, caseine acid 3% and yeast extract 1% and incubated under static condition at 35°C for 48 h. The cultures were aspirated; the tubes were washed twice with distilled water and then stained with 0.25% safranin. Slime production was judged to have occurred and adherent growth to be present if a visible stained film lined the inner wall of the tube. The test was carried out three times in different occasions, each performed in-duplicates. The amount of stained biofilm was macroscopically semiquantitated and the results were registered by semiquantitative form using an estimate grade of slime production as follows: strong (+3), moderate (2+), weak (1+) or absent (0).

Adherence assay

Adherence of the bacterial strains was determined by using a spectrophotometric method described by Christensen *et al.* (1985). The bacterial strains of 18 h stationary culture in TSB were washed, diluted with fresh medium and standardized to contain about 10^5 (CFU/ml). For testing the ability

of E/S or essential oil to prevent microbial adherence (planktonic cells t_o)150 µl of 18 h broth culture and 50 µl of the E/S or the tested oil were mixed in each well of sterile, polystyrene, flat bottomed tissue culture plate (Nunclon, Denmark). In case of the control wells 150 µl of the broth culture was mixed with 50 µl of sterile phosphate buffered saline PBS (pH 7.2). Following 48h of incubation at 35°C, the contents of the culture plates were gently aspirated with a micropipette. The plates were then washed with sterile buffer. Slime and adherent organisms were fixed by incubating them for 1 h at 60°C (Baldassarri *et al.,* 1993) for Gr-ve and by Bouin's fixative for Gr +ve (John *et al*., 1995) and then staining with Hucker crystal violet for 5 min. After washing with water to remove the excess of stain, the plates were dried for 30 min at 37°C., after drying the optical densities (ODs) of stained adherent biofilm were read with Micro ELISA Auto Reader at wave length 492nm for *P. aeruginosa* and 570nm for *St. aureus* and *St. epidermidis.* Adherence measurements were performed in quadruplicate and repeated at least three times, the values were then averaged.

For testing the ability of E/S or essential oil to eradicate the performed biofilm (cellular aggregates t_{24}), the wells were filled with 150 µl for 24h broth cultures of the tested strains then incubated. After incubation, 50 µl of the E/S or the tested oil was added to the tested well, while 50 ml of sterile buffer was added to the control wells. At the same temperature, further incubation for additional 48 h was carried out and the procedure was then completed as described before (Hafez *et al.,* 2002). The percentage of inhibition was expressed as a percentage relative to the corresponding control and was calculated as: 100 – [(O.D. of sample / O.D. of control) x 100]

Statistical analysis

Data were analyzed by unpaired two-tailed Student's *t*-test (Sendecor and Cochran, 1980). The differences between means were considered to be statistically significant at $p<0.05$.

Results and Discussion

Antibacterial assay

The antibacterial activity of the E/S of non-irradiated and irradiated (at 20 Gy) and three essential oils namely, clove, cinnamon and marjoram against certain pathogenic strains was presented in Table 1. In general all the microbial

strains demonstrated resistance to all of the tested substances except marjoram oil which slightly affected the growth of the tested strains (inhibition zone, 6 mm). These results are in agreement with that obtained by Cazander *et al.* (2009[b]) who reported that live maggots from *Lucilia sericata* showed an increase of bacterial growth and instar-1 maggots stimulated more bacterial growth than instar-3 maggots. Also, Park *et al.* (2010) studied the antimicrobial effect of ethyl alcohol extracted fractions obtained from fly maggots *(Musca domestica* L) against MRSA strains and VRE (vancomycin resistant enterococci) 5117 strain and found that the antibacterial activity of hexane, ethylacetate and water layers could not be adequately confirmed. While the butanol fraction showed profound antibacterial activity against the same strains. This result is likely reflecting the difference of antibacterial substance in the different organic solvents which may be due to its different polarities. Other investigators, Arora *et al.* (2011) studied the antimicrobial activity of *Lucilia cuprina* blow fly and found that the E/S had partial bacterial growth inhibition on *St. aureus.* Concerning the antibacterial effect of the tested essential oils, our results were consistent with that obtained by El-Sayed and El-Tablawy (2009) who found that the essential oil of marjoram has antibacterial activity against *P. aeruginosa* but not against *St. aureus* action. While, Hussein *et al.* (2007) found that the essential oil of clove affected the growth of *St. aureus* and *St. epidermidis.* These differences in results may be due to little diffusion properties in the agar or because fresh plants contain active substances which may be affected or disappeared by steps of extraction method (El-Astal *et al.*, 2005). Another explanation; these results would strongly suggested an anti-adherent of the tested oils rather than an antibacterial effect.

TABLE 1. Antibacterial activity of *Lucilia sericata* E/S and essential oils against certain pathogenic bacterial strains.

Tested strain	Zone of inhibition (mm)				
	E/S	E/S*	Clove	cinnamon	marjoram
P. aeruginosa	--	--	--	--	6
St. aureus	--	--	--	--	6
St. epidermidis	--	--	--	--	6

E/S: Excretions and secretions of non-irradiated maggots.
E/S*: Excretions and secretions of irradiated maggots at 20 Gy.

The results in Table 1. indicate that, there is no direct antibacterial effect of E/S *in vitro* even though in clinical observations *Lucilia sericata* maggots therapy has been successful. Therefore, we studied the anti-adherent activity of

E/S as well as the tested essential oils on the selected pathogenic bacterial strains.

Anti-adherent activity of Lucilia sericata* maggot *E/S and essential oils

Slime production and biofilm formation are important factors of pathogenicity in all microorganisms (Hola *et al.,* 2006). The results of the qualitative assessment of slime production indicate that the tested pathogenic strains were positive for production of slime and the grade of production was strong (+3) for *P. aeruginosa,* while it was weak (+1) for *St. aureus* and *St. epidermidis.* The anti-adherent activity of all of the tested substances was evaluated quantitatively through testing their ability to prevent the microbial adherence and eradicate the pre-adhered biofilm (t_{24}) of the tested strains. The results in Table 2. obtained by spectrophotometric method (quantitative method) agreed with that of the visual reading method (qualitative method). The results revealed that the ODs ranging from 0.132 to 0.440. These were a strain-to-strain variation in biofilm formation probably in relation to their individual propensity to adhere to polystyrene surface. All the tested oils and the E/S decreased significantly the ODs of the tested strains except clove oil which increase the OD of *St. epidermidis* significantly.

Our findings were comparable to the published data by Abokamar and Abdelaziz (2003) who observed good anti-adherent activity by using clove, thyme, lemon, cinnamon and peppermint on *Escherichia coli* and *Helicobacter pylori,* while camphor oil showed low anti-adherent productivity on the same strains. Also, El-Sayed and El-tablawy (2009) reported that thyme oil had anti-adherent activity against *E. coil* and *B. cereus*. Concerning the anti-adherent activity of the E/S, Cazander *et al.* (2009[a]) studied the effect of *Lucilia sericata* maggot E/S on the biofilm of *P. aeruginosa* (PAO1) on different biomaterials and found that it prevented and inhibited the biofilm of *P. aeruginosa*.

In Table 2, it was found that strongest biofilm producing strain was *P. aeruginosa.* Therefore the effects of the E/S and the tested oils on the biofilm of *P. aeruginosa* were compared and summarized as in Table 3. The biofilm formation was most affected by clove oil (> 80%), then by marjoram (69%), followed by E/S of non-irradiated maggots (66%). It was less affected by cinnamon oil and E/S of the irradiated maggots (50% and 36%), respectively. It seemed that irradiation reduced the anti-adherent effect of E/S and this may be

due to the effect of ionizing radiation on protein structure. Rao *et al.* (2005) reported that ionizing radiation is known to reduce *Wolbachi*a DNA in worm tissues.

Other investigators Cazander *et al.* (2009[a]) found that the optimum protein concentrations of E/S for biofilm reduction was 80 to 160 µg/ml, whilst van der Plas *et al.* (2008) reported biofilm reduction occurred at protein concentration E/S up to 20 µg per well.

TABLE 2. The anti-adherent activity of E/S of irradiated and non-irradiated *Lucilia sericata* maggot and essential oils against the tested pathogenic strains to prevent bacterial adherence to polystyrene surface.

Tested strains	Adherence measurment (optical densities,ODs)					
	control	**E/S**	E/S*	**clove**	**cinnamon**	**marjoram**
P. aeruginosa	0.44	0.148	0.282	0.073	0.2185	0.138
St. aureus	0.161	0.099	0.095	0.054	0.119	0.127
St. epidermidis	0.132	0.057	0.102	0.142	0.047	0.047

E/S: Excretions and secretions of non-irradiated maggots.
E/S*: Excretions and secretions of irradiated maggots at 20 Gy.
P. values were significant (P<0.05). OD≤0.120: Non- adherent.
0.24≥ODs> 0.12 : Weakly adherent. ODs> 0.24: Strongly adherent.

TABLE 3. Percentage of biofilm of *P. aeruginosa* by the tested maggot E/S and tested oils.

Treatment	Total biofilm	% inhibition
Control	0.440	==
E/S	0.148	66.36
E/S*	0.283	35.68
Clove	0.073	83.41
Cinnamon	0.218	50.45
marjoram	0.138	68.64

E/S: Excretions and secretions of non-irradiated maggots.
E/S*: Excretions and secretions of irradiated maggots at 20 Gy.
Percentage of inhibition calculated using control as reference.

Table 3 also revealed that *P. aeruginosa* biofilm was affected strongly by the E/S of non-irradiated maggots and clove oil. Therefore, they were selected for further study.The ability of E/S of non-irradiated maggots and clove oil to eradicate the performed biofilm (t_{24}) of *P. aeruginosa* was presented in Table 4. The obtained data indicates that the difference in optical density (OD) before and after addition of the E/S and clove oil was statistically significant at p-value 0.0001.The results are in accordance with the previous findings of Cazander *et*

al. (2009a) who found that E/S of *Lucilia sericata* maggot can inhibit and even break down the existing biofilm of *P. aeruginosa* on different biomaterials.

TABLE 4. The anti-adherent activity of E/S of non-irradiated tested maggot and clove oil against *P. aeruginosa* to eradicate pre-adhered biofilm.

Tested strain	Anti-adherent activity			
	E/S		Clove oil	
OD	OD	% inhibition	OD	% inhibition
0.426	0.0183	57.04	0.223	45.31

E/S: Excretions and secretions of non-irradiated maggots.

In conclusion, the E/S of non-irradiated *Lucilia sericata* maggots and clove oil greatly reduce the adherence of *P. aeruginosa* to polystyrene surface and affect its pre-adhered biofilm. We believe this finding may be useful to improve patient care, because biofilm make treatment of infected orthopaedic implants and prostheses more difficult. Detailed studies are recommended to examine the possibility of using E/S or essential oils as a sole or adjuvant therapy for *P. aeruginosa* infections and also their toxicity.

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نقييم مضادات الإلتصاق لإفرازات يرقات اليليوسيليا سيريكاتا المشعة وغير المشعة وبعض الزيوت الأساسية ضد بعض السلالات البكتيرية الممرضة

سهام يوسف الطبلاوى و منى محمد أمين

قسمي البحوث الدوائية و البحوث الصحية الاشعاعية ، المركز القومى لبحوث وتكنولوجيا الإشعاع ، صز بز ٢٩ مدينة نصر ، مصر.

تعتبر قدرة الميكروبات على إنتاج المادة اللزجة عديدة السكريات خارج الخلية من أهم الخواص التى تساعد على إستعمار البكتريا للإنسان وبالتالى قدرتها على إحداث المرض. كما أن زيادة قدرة البكتريا على الإلتصاق على المواد الحيوية يعد مشكلة خطيرة نتيجة لتزايد مقاومة البكتريا للمضادات الحيوية المعروفة.

وفى هذه الدراسة تم إختبار قدرة إفرازات يرقات ليوسيليا سيريكاتا غير المشعة والمشعة عند ٢٠ جراى ، وكذلك بعض الزيوت الأساسية على تثبيط نمو بعض العزلات البكتيرية الممرضة (السودوموناس إيروجينورا ، إستافيلوكوكاس أورياس وإستافيلوكوكاس ابيديرميدس) وكذلك على منع قدرتها على الإلتصاق.

وقد أثبتت النتائج أن جميعها لم يكن لها تأثيرا واضحا كنشاط مضاد للبكتيريا على العزلات البكتيرية محل الدراسة فيما عدا زيت نبات البردقوش الذى كان له تأثيرا محدودا. إلا أن إفرازات يرقات ليوسيليا سيرسكاتا والزيوت الأساسية المختبرة تميزت بقدرتها على منع إلتصاق العزلات البكتيرية المختبرة.

وأوضحت النتائج أن أعلى قدرة على منع إلتصاق السومودوموناس إيروجينوزا كانت مع زيت القرنفل (أكثر من ٨٠٪) وأن قدرة إفرازات اليرقات غير المشعة كانت أعلى من مثيلتها المشعة عند جرعة ٢٠ جراى (٦٦.٣٦ ، ٣٥.٦٨) ٪ على التوالى. فى حين أوضحت النتائج أن قدرة إفرازات اليرقات غير المشعة على تقليل الإلتصاق المتكون لنفس العزلة البكتيرية كانت أعلى من قدرة زيت القرنفل (٥٧ ، ٤٥) ٪ على التوالى.