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EVALUATION OF ADDING ETHYLENE DIAMINE TETRA-ACETIC ACID (EDTA) TO IODOPHOR ON THEIR MICROBICIDAL EFFICACY

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ABSTRACT

The antimicrobial activity of iodophor (I) was investigated before and after adding of ethylenediaminetetraacetic acid (EDTA) (30 mg/mL) in quantitative suspension test and surface disinfectant test against Candida albicans, Escherichia coli ,Salmonella typhimurium and Staphylococcus aureus.The results showed that iodophor with EDTA was significantly more effective than iodophor, EDTA and control .The most significant time was between 1 and 5h and in the strain of Staphylococcus aureus. Candida albicans was the most resistant ,followed by Salmonella typhimurium, then Escherichia coli and the most sensitive was Staphylococcus aureus.

INTRODUCTION

The antimicrobial activity of iodine is through its reaction with cellular enzymes ***(Ramesh et al., 2002)***.Elemental iodine is not very soluble in water; however, this problem is alleviated by adding complexing agents to form iodophors. An iodophor is a complex of elemental iodine and a complexing agent or carrier, which increases the solubility of the iodine and provides a sustained-release reservoir of iodine for germicidal activity ***(Boddie and Nickerson, 1997)***. The development of new technological methods enables the concentration of

free iodine to increase the speed of germicidal action against microorganisms ***(Bonewitz et al., 1988).*** The antimicrobial action of iodine is rapid, even at low concentrations, but the exact mode of action is unknown. Iodine rapidly penetrates into microorganisms ***(Chang, 1971)*** and attacks key groups of proteins (in particular the free-sulfur amino acids cysteine and methionine ***(Gottardi, 1991; Kruse, 1970)***, nucleotides, and fatty acids ***(Apostolov, 1980; Gottardi, 1991)***, which culminates in cell death ***(Gottardi, 1991).***

EDTA is a safe, economical metal chelator which sequesters divalent cations (notably Ca^{2+} and Mg^{2+}) that contribute to the stability of the outer membrane of gram-negative bacteria by providing electrostatic interactions with proteins and lipo-polysaccharides ***(Vaara, 1999).*** Furthermore, the EDTA releases a large proportion of gram-negative lipo-polysaccharides from the outer membrane and exposing hydrophobic phospholipids that increases the susceptibility to hydrophobic and cell wall degrading agents ***(Helander et al., 1997 and Walsh et al., 2003).*** The presence of EDTA can enhance Hf-1 (a novel antibacterial peptide from the larvae of the housefly (*Musca domestica*) in medium and orange juice) activity against Gram-negative bacteria ***(Hou et al.,2007)***. It was recently demonstrated that 30 mg/mL EDTA, when used as a component in an antimicrobial solution, is rapidly and synergistically active in eradicating staphylococcal and *Candida* organisms embedded in biofilm ***(Raad et al., 2007)***. Therefore in the present study, we investigated the possibility of incorporating EDTA and disinfectant (Iodophor) as an effective method against major pathogens including yeast *(Candida albicans)*, gram negative bacteria *(Escherichia coli ,Salmonella typhimurium)* and gram positive bacteria *(Staphylococcus aureus)*.

MATERIALS AND METHODS

1. MATERIALS:

1. 1. Microorganisms

Candida albicans, Escherichia coli ,Salmonella typhimurium that was recommended as a test strain in the council of european nation (CEN) test ***(Council of Europe, 1987)*** and *Staphylococcus aureus* were obtained from department of microbiology at the faculty of veterinary medicine, University of Kafr El-Shaekh. *Escherichia coli ,Salmonella typhimurium* and *Staphylococcus aureus* were initially cultured in nutrient broth at 37 °C for 8 h. While, in case of *Candida albicans,* was initially cultured in Sabouraud broth at 37 °C for 8 h. The microbs were suspended in a solution of 0.85% (w/w) NaCl for subsequent experiments. The initial concentrations of the undiluted suspensions ranged from 5 X $10^7$to 3 X 10^8cfu/ml. ***(Susan Ewart et al., 2001).***

1.2. Disinfectants

1.2. 1-I odosan 30

(A product from Ewabo,Germany), contains total of 300g/L polyethoxyeminoethanol /[nonylphenoxypolyethoxyethanol iodine complex (at 10% of active iodine)and 198 g/L phosphoric acid.The recommended dose is 1.5 ml/L.

1.2. 2- EDTA(30mg/ mL) ***(Raad et al.,2007).***

Ethylenediaminetetraacetic acid (EDTA) (30mg/ mL). and the pH of EDTA is 7.2.

1.3. Neutralizer:

The neutralizer (sodium thiosulphate 0.5%) for iodophor, was used in recovery broth medium according to ***(Reem-Dosoky et al., 2000).***

2. METHODS

2.1. Quantitative suspension test:

An inoculum of microbial suspension in saline, was added to 5ml of tested disinfectant solution (EDTA, iodophor and iodophor with EDTA). After a given exposure time (5 min, 10 min and 15 min). A loopful from the mixture was cultured for surviving organisms. Plates were incubated at $37^{O}C$ for 48 h and CFU/ml determined.

2.2. Practical test (Surface disinfection test):

The test surface (ceramic tiles), was contaminated with a standardized inoculum of each of test microorganisms (10^{8} cfu/ ml), and dried. Then 0.4 l/m of each disinfectant was applied. Each hour, the number of survivors is determined by the use of control series in which the disinfectant was substituted by distilled water; from the comparison of the survivors in this control series with the test series, the reduction is determined quantitatively. Swabs were transferred to 4 ml of 1% peptone broth tubes with neutralizer. Then inoculated in nutrient agar and MacConkey agar for bacteria and sabouraud s agar in *Candida albicans* and incubated at 37 °C for (24 h and 48h in case of bacteria and yeast respectively), at which time the cell number was calculated by the plate count method.

The antimicrobial activity or removal efficiency of disinfectant for intact cells was defined as:

$$\text{Removal efficiency (\%)} = \frac{(\text{initial cell number} - \text{cell number after treatment})}{\text{initial cell number}} \times 100\%$$

(Hassabel-Naby and El-Midany, 2005).

2.3. Statistical analysis:

All values are reported as means of three determinations in duplicate experiments. Analysis of variance (ANOVA) was conducted using the general linear model (GLM) procedure of the Statistical Analysis System (SAS 8.2, SAS Institute Inc., Cary, NC, 2000). The least significant difference (LSD) procedure (Student *t*-test) was used to compare the means and significant mean differences among the treatments and treatment combinations and determined at $p < 0.05$.

RESULTS

EDTA(30 mg/mL), was shown to enhance the activity of iodophor against microbs in a time-dependent fashion. The effectiveness of iodophor with and without EDTA, was evaluated in relation to the survivability of *Candida albicans, Escherichia coli ,Salmonella typhimurium* and *Staphylococcus aureus* in saline. First in quantitative suspension test, the reduction was greatest in the presence of EDTA for 15 min compared to the other exposure times as follow: *Candida albicans*(45%; 1.44 log CFU/mL), *Escherichia coli* (99%; 2.78 log CFU/mL), *Salmonella typhimurium* (70%; 2.08 log CFU/mL) and *Staphylococcus aureus* (100%; (< 0.15 log CFU/mL), (Table 1 and 2). The results of this study provide evidence that EDTA is potentially useful as additive to iodophor in disinfection.Second in practical test (Surface disinfection test), During the first phase (from 1 to 4 h) of the disinfection.EDTA was also shown to enhance significantly the activity of Iodophor against *Staphylococcus aureus* especially after 2 hours (Tables 3 and4). Third in practical test (Surface disinfection test), During the second phase (from 5 to 8 h) of the disinfection. EDTA was also shown to enhance significantly the activity of Iodophor against *Candida albicans* especially at 6 hours(Tables 5 and6).

Table (1): Effect of EDTA(30 mg/mL), iodophor (1.5 ml/L) and their combinations on the microbial load 10^{8} cfu/ ml in quantitative suspension test expressed by(log CFU/mL).

Disinfectants	EDTA			IODOPHOR			IODOPHOR &EDTA		
Time in minutes	5	10	15	5	10	15	5	10	15
Candida albicans	1.05± 0.1^{g}	1.05± 0.1^{g}	1.05± 0.1^{g}	1.05± 0.1^{g}	1.05± 0.1^{g}	1.31 ± 0.3^{f}	1.05± 0.3^{g}	1.18± 0.3^{fg}	1.44± 0.2^{f}
Escherichia coli	1.05± 0.3^{g}	1.44± 0.2^{f}	2.08± 0.3^{d}	1.56± 0.3^{ef}	2.08± 0.3^{d}	2.78 ± 0.1^{b}	2.08± 0.3^{d}	2.60± 0.1^{bc}	2.78± 0.1^{b}
Salmonella typhimurium	1.05± 0.3^{g}	1.44± 0.2^{f}	2.08± 0.3^{d}	1.44± 0.2^{f}	1.44± 0.2^{f}	2.08 ± 0.3^{d}	1.56± 0.3^{ef}	1.83± 0.3^{e}	2.08± 0.2^{d}
Staphylococcus aureus	1.05± 0.3^{g}	1.44± 0.3^{f}	2.60± 0.3^{bc}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}

Means with superscript different letter(s) differ significantly at ($P < 0.05$).

Table (2): Effect of EDTA(30 mg/mL), iodophor (1.5 ml/L) and their combinations on the microbial load 10^{8} cfu/ ml in quantitative suspension test expressed by removal efficiency%.

Disinfectants	EDTA			IODOPHOR			IODOPHOR &EDTA		
Time in minutes	5	10	15	5	10	15	5	10	15
Candida albicans	30± 0.1^{g}	30± 0.1^{g}	30± 0.1^{g}	30± 0.1^{g}	30± 0.1^{g}	40± 0.1^{f}	30± 0.2^{g}	35± 0.1^{fg}	45± 0.1^{ef}
Escherichia coli	30± 0.2^{g}	45± 0.4^{ef}	70± 0.3^{cd}	50± 0.3^{de}	70± 0.2^{cd}	90± 0.3^{ab}	70± 0.3^{cd}	90± 0.4^{ab}	99± 0.1^{a}
Salmonella typhimurium	30± 0.2^{g}	45± 0.3^{ef}	70± 0.3^{cd}	45± 0.1^{ef}	45± 0.1^{ef}	60± 0.3^{d}	50± 0.3^{de}	60± 0.3^{d}	70± 0.3^{cd}
Staphylococcus aureus	30± 0.2^{g}	45± 0.3^{ef}	90± 0.4^{ab}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}

Means with superscript different letter(s) differ significantly at ($P < 0.05$).

Table (3): Effect of EDTA(30 mg/mL), iodophor (1.5 ml/L) and their combinations on the microbial load 10^{8} cfu/ ml in Practical test (Surface disinfection test) during the first phase (from 1 to 4 h) of the disinfection expressed by (log CFU/mL).

Disinfectants	EDTA				IODOPHOR				IODOPHOR &EDTA			
Time in minutes	1	2	3	4	1	2	3	4	1	2	3	4
Candida albicans	1.05± 0.2^{g}	1.05± 0.2^{g}	1.05± 0.1^{g}	1.05± 0.1^{g}	1.57± 0.2^{ef}	1.71± 0.1^{e}	1.83± 0.1^{e}	2.21± 0.2^{d}	1.83± 0.3^{e}	2.08± 0.1^{d}	2.34± 0.2^{cd}	2.47± 0.1^{c}
Escherichia coli	1.05± 0.2^{g}	1.18± 0.2^{f}	1.31± 0.1^{f}	1.44± 0.1^{f}	1.71± 0.2^{e}	1.96± 0.2^{de}	2.34± 0.1^{cd}	2.47± 0.1^{c}	1.96± 0.2^{dc}	2.21± 0.2^{d}	2.60± 0.3^{bc}	< 0.15 ^{a}
Salmonella typhimurium	1.05± 0.1^{g}	1.05± 0.1^{g}	1.05± 0.2^{g}	1.18± 0.2^{fg}	1.71± 0.1^{e}	1.83± 0.2^{e}	2.21± 0.2^{d}	2.47± 0.2^{c}	1.83± 0.2^{e}	1.96± 0.1^{de}	2.34± 0.1^{cd}	2.60± 0.1^{bc}
Staphylococcus aureus	1.31± 0.3^{f}	1.83± 0.2^{c}	2.08± 0.1^{d}	2.21± 0.2^{d}	1.83± 0.3^{c}	2.08± 0.2^{c}	2.34± 0.1^{cd}	2.60± 0.1^{bc}	2.34± 0.3^{cd}	< 0.15 ^{a}	< 0.15 ^{a}	< 0.15 ^{a}

Means with superscript different letter (s) differ significantly at ($P < 0.05$).

Table (4): Effect of EDTA(30 mg/mL), iodophor (1.5 ml/L) and their combinations on the microbial load 10^{8} cfu/ ml in Practical test (Surface disinfection test) during the first phase (from 1 to 4 h) of the disinfection expressed by removal efficiency%.

Disinfectants	EDTA				IODOPHOR				IODOPHOR &EDTA			
Time in minutes	1	2	3	4	1	2	3	4	1	2	3	4
Candida albicans	30± 0.1^{g}	30± 0.1^{g}	30± 0.2^{g}	30± 0.1^{g}	50± 0.1^{de}	55± 0.1^{de}	60± 0.1^{d}	75± 0.3^{cd}	60± 0.3^{d}	70± 0.3^{cd}	80± 0.2^{bc}	85± 0.1^{b}
Escherichia coli	30± 0.1^{g}	35± 0.1^{fg}	40± 0.1^{f}	45± 0.2^{ef}	55± 0.3^{de}	65± 0.2^{d}	80± 0.1^{bc}	85± 0.2^{b}	65± 0.3^{d}	75± 0.2^{cd}	90± 0.3^{ab}	100^{a}
Salmonella typhimurium	30± 0.1^{g}	30± 0.2^{g}	30± 0.1^{g}	35± 0.2^{fg}	55± 0.2^{de}	60± 0.1^{d}	75± 0.3^{cd}	85± 0.2^{b}	60± 0.1^{d}	65± 0.2^{d}	80± 0.3^{bc}	90± 0.1^{ab}
Staphylococcus aureus	40± 0.3^{f}	60± 0.2^{d}	70± 0.2^{cd}	75± 0.1^{cd}	60± 0.2^{d}	70± 0.3^{cd}	80± 0.2^{bc}	90± 0.2^{ab}	80± 0.1^{bc}	100^{a}	100^{a}	100^{a}

Means with superscript different letter (s) differ significantly at ($P < 0.05$).

Table (5): Effect of EDTA(30 mg/mL), iodophor (1.5 ml/L) and their combinations on the microbial load 10^{8} cfu/ ml in Practical test (Surface disinfection test) during the second phase (from 5 to 8h) of the disinfection expressed by (log CFU/mL).

Disinfectants	EDTA				IODOPHOR				IODOPHOR &EDTA			
Time in minutes	5	6	7	8	5	6	7	8	5	6	7	8
Candida albicans	1.05± 0.1^{g}	1.05± 0.2^{g}	1.05± 0.2^{g}	1.15± 0.3^{fg}	2.34± 0.1^{cd}	2.47± 0.1^{c}	2.60± 0.1^{bc}	< 0.15^{a}	2.60± 0.1^{bc}	2.78± 0.1^{b}	2.78± 0.1^{b}	< 0.15^{a}
Escherichia coli	1.56± 0.1^{ef}	1.83± 0.1^{e}	2.08± 0.1^{d}	2.21± 0.1^{d}	2.60± 0.1^{bc}	2.78± 0.1^{b}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}
Salmonella typhimurium	1.31± 0.2^{f}	1.44± 0.1^{f}	1.71± 0.1^{e}	1.83± 0.1^{e}	2.47± 0.1^{c}	2.60± 0.1^{bc}	2.78± 0.1^{b}	< 0.15^{a}	2.78± 0.1^{b}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}
Staphylococcus aureus	2.35± 0.1^{cd}	2.47± 0.1^{c}	2.60± 0.1^{bc}	< 0.15^{a}	2.60± 0.1^{bc}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}	< 0.15^{a}

Means with superscript different letter (s) differ significantly at ($P < 0.05$).

Table (6): Effect of EDTA(30 mg/mL), iodophor (1.5 ml/L) and their combinations on the microbial load 10^{8} cfu/ ml in Practical test (Surface disinfection test) during the second phase (from 5 to 8h) of the disinfection expressed by removal efficiency%.

Disinfectants	EDTA				IODOPHOR				IODOPHOR &EDTA			
Time in minutes	**5**	**6**	**7**	**8**	**5**	**6**	**7**	**8**	**5**	**6**	**7**	**8**
Candida albicans	30± 0.1 ^{g}	30± 0.2 ^{g}	30± 0.2 ^{g}	33± 0.2 ^{fg}	80± 0.1^{bc}	85± 0.1^{b}	90± 0.1^{ab}	100 ^{a}	90± 0.2^{ab}	99± 0.1^{a}	99± 0.1^{a}	100 ^{a}
Escherichia coli	50± 0.4 ^{de}	60± 0.3^{d}	70± 0.2^{cd}	75± 0.1^{cd}	90± 0.1^{ab}	99± 0.1^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}
Salmonella typhimurium	40± 0.2 ^{f}	45± 0.3 ^{ef}	55± 0.3 ^{de}	60± 0.2^{d}	85± 0.3^{b}	90± 0.1^{ab}	99± 0.1^{a}	100 ^{a}	99± 0.1^{a}	100 ^{a}	100 ^{a}	100 ^{a}
Staphylococcus aureus	80± 0.1^{bc}	85± 0.1^{b}	90± 0.1^{ab}	100 ^{a}	90± 0.1^{ab}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}	100 ^{a}

Means with superscript different letter (s) differ significantly at ($P < 0.05$).

DISCUSSION

The results of this current study showed that EDTA enhanced the activity of iodophor against Candida albicans ,Salmonella typhimurium ,Escherichia coli and Staphylococcus aureus on the contaminated surfaces especially after an exposure time of 2–4 h. The combination of iodophor and EDTA demonstrated superior inhibitory activity against Candida albicans (after 8 h of exposure), Salmonella typhimurium (after 6 h of exposure), Escherichia coli (after 4 h of exposure) and Staphylococcus aureus (after 2h of exposure) contaminated ceramic tiles compared with iodophor or EDTA alone. EDTA alone inhibited the Gram-positives pathogens tested in accordance with ***Hou et al.,(2007)***. Although EDTA alone failed to inactivate Candida albicans completely it significantly enhanced the efficacy of iodophor to inhibit Candida albicans formation through its inhibitory effect on filamentation ***(Ramage et al., 2007)***. EDTA disrupts microbial biofilm through its chelation of calcium, magnesium and iron, which are essential components of the biofilm matrix ***(Banin et al., 2006; Percival et al., 2005; Raad et al., 2007; Shanks et al., 2005)***. Staphylococcus aureus was more sensitive to disinfectant solutions than the other three pathogens tested. Iodophor alone reduced Staphylococcus aureus population by 60% after 1 h. The EDTA enhanced the activity of iodophor against these pathogens. The less inhibitory activities against Gram-negative E. coli and S. typhimurium might be related with the structure of the cell membrane. This expectation was due to that several other studies have shown that the inhibitory effect of phenolic compounds from natural extracts are more potent to Gram-positive bacteria than Gram-negative ***(Beuchat and Golden, 1989).*** This pathogenic capability of Gram-negative bacteria is usually associated with the

presence of lipopolysaccharide (LPS) layer, which might be involved in reducing the sensitivity of these bacteria., suggesting that E. coli was more susceptible to disinfection than Salmonella. Gram-negative bacteria are generally less susceptible to biocides because of their complex cell wall, which is composed of the inner cytoplasmic membrane (CM) and associated efflux pumps, peptidoglycan, and an outer membrane (OM) with associated LPS components. The OM also contains hydrophilic channels, porins that regulate the passage of solutes ***(Denyer and Maillard, 2002)***.The main component responsible for the impermeability of the OM is the LPS. Change in cell wall expression or structure leads to increased nonsusceptibility of gram-negative bacteria to biocides ***(Lambert, 2002)***. LPS is the primary barrier to the penetration by hydrophobic molecules to the phospholipids and to the cell interior. In addition, hydrophilic molecules pass readily into gram negative bacteria but exposure of E. coli to biocides results in porin loss and subsequent decreased susceptibility to biocides ***(Denyer and Maillard, 2002)***.This combination was found to be a good disinfectant that will be with other rotated disinfectants -to prevent resistance development- a substitute to the aldehydes, especially formaldehyde as were evaluated in the United States by the ***(Department of Labor Federal Register 1987)*** as a dangerous disinfectant for their harmful effects on the human body.

CONCLUSION

In conclusion, the combination of EDTA with Iodophor demonstrated superior inhibitory activity against tested microorganisms embedded in biofilm compared with Iodophor and EDTA alone. *Candida albicans* was the most resistant ,followed by *Salmonella typhimurium*, then *Escherichia coli* and the most sensitive was *Staphylococcus aureus*.

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تقييم إضافة حمض إثيلين ثنائي أمين رباعي الخليك (إديتا) على فاعلية الأيودوفور لقتل الميكروبات.

تم اختبار قدرة الأيودوفور قبل وبعد إضافة حمض إثيلين ثنائي أمين رباعي الخليك (إديتا) بكمية 30 ملجم لكل مللي ليتر وذلك باستخدام اختبار المحلول العيارى و اختبارتطهير الأسطح ضد اكانديدا البيضاء والميكروب القولونى والسالمونيلا تايفيموريوم والميكروب العنقودى الذهبى.

أثبتت التجارب أن إضافة الاديتا قد زادت من قدرة الأيودوفور زيادة معنوية عن تأثيره و كذلك تأثير الاديتا و المحلول الضابط. وخاصة في فترة مابين الساعة الأولى والخامسة وخاصة على الميكروب العنقودى الذهبى. وكانت الكانديدا البيضاء أكثر الميكروبات مقاومة يليها السالمونيلا تايفيموريوم فالميكروب القولونى و كان الميكروب العنقودى الذهبى هو الأكثر تأثرا.