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Copper Enhancement of Dettol Lethality to *Candida albicans*

Mohamed M. Ghareb[1], Mohamed A. Elhefnawy[1], Mohamed Ibrahim[2], Azza M. Soliman[1] and Awad A. Elshoura[1]*

[1]Botany Department, Faculty of Science, Menofia University.
[2]Botany Department, Faculty of Science, Cairo University.

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The overall objective in this study is to explore the exact lethal dose of dettol as an approach to minimize the total amount of unnecessary application. The addition of copper increased the lethality effect of dettol. Different dettol concentrations (10 to100ppm) were used to study the effect of dettol on the pathogenic yeast *Candida albicans* growth profile using Malt Extract Agar (MEA) and Malt Yeast Glucose Peptone (MYGP) media. The results showed that the fungal growth was inhibited by 24.6% when dettol was used in concentration 30ppm, but with addition of copper by 0.2 mM the fungal growth inhibition increased to 49.2%. Also the addition of 0.1mM as $CuCl_2$ to growth medium with 50 ppm of dettol resulted in completely elimination the growth of *C. albicans* compared to dettol alone (92.7% inhibition). From this study it was concluded that the lethal dose of dettol could be reduced to *C. albicans* by 99% of the common practice dose that is used. The germ tube formation of *C. albicans* was used to detect the effect of different concentrations of dettol, Cu and dettol with Cu. The results showed that the highest stress was found at treatment with dettol and Cu with highest number of germ tube formation 500±3.94 germ tube in 100 ml culture.

Key words: *Candida albicans*, Dettol, Chloroxylenol, Copper.

Candida albicans is a normal flora of the human and animal digestive tract of the family Saccharomycetaceae, currently the most common species causing candidaemia **(**Pfaller and Diekema, 2002; Tortorano *et al.*, 2004). It is the commonest fungus of medical importance. Candidiasis can be a superficial infection of skin, nails or mucous membrane with the yeast form of the fungus, causing mild inflammation (Larone, 2002) *C. albicans* is commonly found at low levels among the normal oral flora, but its overgrowth in immuno-compromised individuals or following broad-spectrum antibiotic therapy leads to oropharyngeal candidiasis (Sanglard, and Odds 2002). The *C. albicans* is the most common cause of candidiasis, which can be acute, sub-acute or chronic infection involving any part of the body. Increase use of dettol which is one of phenolic disinfectant that threat the environment and the public health especially after the increased public awareness of the daily common health practices. The toxic effect of copper on fungal cells has long been of interest to mycologists as a result of its high value as fungicide. Copper is one of the metals which at low concentration can exhibit toxicity (Morgan and Stumm, 1991). Copper can interact with fungal cells, and be accumulated by physico-chemical mechanism and transport systems of varying specificity (Gadd, 1988). There have been many studies on the inhibition of spore germination by copper and some other heavy metals ions at various concentrations (Gadd, 1981). Ochiai (1987) and Gadd (1993) reported that the toxic effect of copper and other heavy metals is related to the strong

* To whom all correspondence should be addressed.
E-mail: shoura71@hotmail.com

coordinating abilities of heavy metals include blocking of functional groups of important molecules like enzymes and poly nucleotides, transport systems for essential nutrients and ions and substitution of essential ions from cellular sites in addition to disruption of cellular and organelles membrane integrity. However, at high concentrations, copper interacts with cellular nucleic acids and enzyme active sites (Stohs and Bagchi, 1995). Thus, copper is a potent inhibitor of fungal growth and is a main component of several fungicides. But the presence of high concentration of copper in environment promotes the selection of microorganisms possessing genetic resistance (Cervantes and Gutierrez, 1994). Dettol as one of the phenolic compounds may destroy plasma membranes and denature proteins **(**Michalowicz and Duda, 2007**)**. Phenol is rapidly absorbed following inhalation and ingestion (Ferner *et al*., 1999, ATSDR, 1998). The skin is thought to be the primary route of entry during occupation exposure (ATSDR, 1998), so once absorbed phenol is rapidly distributed to all tissues in animals (IPCS, 1994a and1998, ATSDR, 1998). The highest peak concentrations were found in liver although both phenol and metabolites were also detected in the lungs, CNS, spleen, kidney, adrenal and thyroid gland, depending on the animal species (DEFRA, 2003). These compounds are widely represented in natural environment. The majority of phenols in the atmosphere however is from anthropogenic activity (IPCS, 1994 a,b). Most of the phenols show negative action towards living organisms including humans. The presence of these compounds in the biosphere led to their transformation undergoing under the influence both abiotic and abiotic factors (Michalowicz and Duda, 2007). The transformation processes most often lead to the total degradation (mineralization) of these compounds. The possibility of phenols degradation by microorganisms is due to creation of enzymes capable to transform phenolic xenobiotics and use them as the source of aliment and energy. Both in the environment and in living organisms some transformations may lead to creation of most harmful products of these processes **(**Michalowicz and Duda, 2007). Dettol is widely used in clinical disinfectant applications to avoid microbial infections especially *Candida*. The increase use of disinfectant due to the increase public awareness of the daily common health practices. This sharply increases in the consumption of such disinfectant, increases an unexpected environmental hazardous impact due to the large amount of such chemicals in sewages and subsequently in the ecosystem. Among these disinfectants is dettol which is one of phenolic compounds that threat the environment and the public health. The recorded application overdose of dettol in the common practice which ranged from 2500–5000 ppm (1:20-1:10 dilution) needs to be reviewed as this application dose along with the sharply increased consumption causes a growing threat to the environment. Also, there is a fear that repeated excessive use of these disinfectants may lose its main function in killing microbes or at least prevent the harmful impact of these microbes by stopping its activities. This fear is caused by the possibility of gaining these microbes acquired resistance to convey from one generation to another generation evolving new generations of harmful microbes resistant to these disinfectants. Therefore the overall objective is to explore the exact lethal dose of dettol as an approach to minimize the total amount of unnecessary application. Also to explore the addition of another compound such as (copper), that may increase the lethality effect of dettol as a second approach for such minimization of dettol application.

MATERIALS AND METHODS

The organism

Candida albicans was obtained from the Regional Center of Fungi (Al-Azhar University) to represent one of the most common pathogenic fungi in medical vicinities. *Candida albicans* was maintained on Malt Extract Agar medium (MEA) and Malt Yeast Glucose Peptone medium (MYGP).

Chemicals

Dettol was purchased from Pharmaceutical stores in Jeddah, Saudi Arabia as commercially formulated product. It consisted of Chloroxylenol 4.8% (v/v), Olium pine Aromaticum 9% (v/v), denature spirits 11.3% (v/v), and Sapovegetalis 5% (v/v). The used dilutions by part per milion (ppm) 10 ppm, 20ppm, 30ppm, 40ppm and 50 ppm. The active material is Chloroxylenol (4-chloro-3, 5-dimethylphenol).

The used copper is a solution of copper chloride dihydrate ($CuCl_2.2H_2O$) with a final concentration of 0.1, 0.2, 0.3, 0.4, and 0.5 mM.

Mixtures of dettol and copper were prepared to give final concentration of 10, 20, 30, 40 and 50 ppm dettol with 0.1 and 0.2 mM copper. This mixing process resulted in 10 mixture solutions.

The media

The MYGP media was mainly used for culturing the yeast and was prepared by dissolving 3.0 g yeast extract, 3.0 g malt extract, 5 g gelatin, 10 g peptone dextrose and 15 g agar in one liter of distilled water. The pH was then adjusted to 5.7. Also Malt Extract Agar (MEA) was used in culturing the C*andida albicans* that prepared by adding 12.75 g maltose, 2.75 g dextrin, 2.35 g glycerol, 0.78 g peptone, 15.0 g agar in one liter of distilled water and the pH was adjusted to 5.6.

Germ tube formation of *candida albicans*

Adjusting cell concentration of yeasts, washed *Candida albicans* was inoculated into a culture flask in human(or rabbit) blood serum and incubated at 37-39°C for 1-3 h. the germ tube was observed and counted by using haemacytometer as described by Kim *et al.* (2002)

Growth monitor

The growth of *Candida albicans* was monitored using the Optical density (O.D) of 3-day culture at 550 nm using a spectrophotometer of the yeast cells (spectro 23 RS) to study the effect of dettol and copper chloride.

Resistance of the successive generations of *Candida albicans* to dettol

To detect the fungal resistance to dettol in the successive generations of *Candida albicans*, the culture was monitored at the 3rd day of growth (first generation) and transferred at the same day to a new medium (second generation) with the same dettol concentration and so on till the 4th generation.

RESULTS

Effect of dettol on growth of *Candida albicans*

The O.D of *Candida albicans* cultures was used as an indicator of the growth. Figure1 shows that the higher the dettol concentration the lower the growth of *C. albicans*. Although low effect of dettol on *C. albicans* was noticed at 10 ppm and 20 ppm (0 and 4.1% growth reduction respectively) the % reduction increased from 20.7% to 92.3% by increasing the dettol concentration from 30 ppm to 50 ppm respectively.

Effect of copper chloride on the growth of *Candida albicans*

The hypothesis of the present study was the effect of dettol could be enhanced by the addition of copper chloride to increase the fungicidal effect of dettol. Fig.2 shows that the increasing in copper concentrations causes a decrease in the growth of *Candida albicans*. The higher concentration of copper chloride caused the higher reduction percent of the growth. At 0.1 mM ($CuCl_2 \cdot 2H_2O$) the percent reduction was 30.11% where at 0.2 mM ($CuCl_2 \cdot 2H_2O$) it reached 43.18%.The maximum percent reduction (93.75%) was at 0.5 mM (Fig.2). As a result of this experiment we decided to use the concentration of 0.2 mM of copper chloride which gives a high % reduction with minimal concentration of copper chloride.

Effect of addition of copper to dettol on the growth and germ tube formation of *Candida albicans*

A decrease in the absorbance of *Candida albicans* cultures as a result of adding copper to

Table 1. Effect of different concentrations of dettol amended with 0.1 and 0.2 mM copper chloride on the Optical density (O D) of *Candida albicans* (absorbance at 550 nm). Each value is the mean of three readings ± SE of the mean

0.2		0.1		0.0		Cu conc.(mM)
Red. %	O D	Red. %	O D	Red. %	O D	Dettol (ppm)
17.9	1.47±0.03	7.8	1.65±0.04	0.0	1.79±0.06	0.0
25.0	1.36±0.03	12.8	1.56±0.04	3.9	1.72±0 .05	10
35.8	1.15±0.02	17.9	1.47±03	9.5	1.62±0.04	20
49.2	0.91±0.01	34.6	1.17±0.02	24.6	1.35±0.02	30
75.9	0.43±0.01	63.1	0.66±0.02	53.6	0.83±0.02	40
100	0.0±0.0	100	0.0±0.0	92.7	0.13±0.01	50

dettol was detected (Figure 3). The % reduction calculated in Table (1) is calculated by comparing each number in the table with the zero dettol and zero copper (Control) (1.79 O.D). The % reduction was decreased from 3.9% due to 10ppm dettol and from 7.8% due to 0.1 mM copper to 12.8% due to addition of 0.1 mM copper to the 10ppm dettol. This increased reduction was observed in all treatments in this experiment. The maximum reduction percentage (100%) was observed with the addition of 0.1 and 0.2 mM copper to 50ppm dettol.

The germ tube formation of *Candida albicans* was used to detect the effect of different concentrations of dettol, 0.2 mM Cu and dettol aided with 0.2 mM Cu on *Candida albicans*. Table (2) showed that the higher the stress over the organism the higher the number of germ tubes. This positive correlation is not only related to the number of germ tubes but also related to the increasing number of cells going into the decline phase. So in fact the real effect of dettol and copper is the acceleration of the growth profile toward the decline phase and the increasing number of cells in shorter time compared with the normal growth profile which will end with the higher cells number but in a longer time (Figure 4).

Since germ tube formation is one of the most characteristic features of *Candida*, therefore the obtained isolate of Candida albicans was examined for its ability to form germ tube according to Kim *et al.*, (2002). This examination proved that the obtained isolate has the ability to form germ tube. The germ tube formation of *Candida albicans* was used to detect the effect of different concentration of dettol, 0.2 mM Cu and dettol aided with 0.2 mM Cu on *Candida albicans*. Table (2) showed that the higher the stress over the organism the higher the number of germ tubes.

Resistance of successive generations of *Candida albicans* to dettol

To detect the fungal resistance to dettol in the successive generations of *Candida albicans*, the culture was monitored over 3 days growth (first generation) and transferred to a new medium (second generation) with the same dettol concentration and so on till the 4th generation. *C. albicans* showed no change in their growth in the generation level at zero dettol concentration, while clear increase was observed in such growth with the presence of dettol in the successive generations compared with the first generation (Table 3). At 10ppm dettol the second generation showed an increase by 3.55% compared with the first generation where the 3rd and 4th generations showed 4.73% and 8.28% respectively. This

Table 2. Effect of dettol alone (20ppm), copper alone (0.2 mM) and dettol (20ppm) amended with copper (0.2 mM) on the number of germ tubes / 100 ml *Candida albicans* cultures. Each value is the mean of three readings ± SE of the mean.

Dettol and Copper Concentration	No. of germ tube in 100 ml culture
Zero Dettol Zero Copper	300±1.33
20 ppm Dettol	430±2.56
0.2 mM Copper	370±0.89
20 Dettol + 0.2 Cu	500±3.94

Table 3. Effect of different concentrations of dettol (ppm) on the culture optical density (OD) (absorbance at 550nm) of four successive generations of *Candida albicans*. Each value is the mean of three readings ± SE of the mean.

Dettol Concentration (ppm)	G 1	G 2		G 3		G 4	
	O D	O D	increase%	O D	increase%	O D	increase%
0.0	1.79±0.06	1.82±0.05	1.68	1.83±0.04	2.23	1.81±0.06	1.11
10	1.69±0.04	1.75±0.06	3.55	1.77±0.03	4.73	1.83±0.05	8.28
20	1.66±0.04	1.75±0.05	5.42	1.76±0.04	6.02	1.80±0.04	8.43
30	1.53±0.03	1.63±0.04	6.54	1.65±0.04	7.84	1.67±0.04	9.15
40	0.93±0.03	1.30±0.03	39.78	1.35±0.3	45.16	1.39±0.02	49.46
50	0.22±0.01	0.30±0.01	36.36	0.33±0.02	50	0.35±0.01	59.09

increase was also observed at 20 and 30 ppm dettol with 5.42% and 6.54% increase respectively in the 2nd generation and reached 8.43% and 9.15% increase respectively in the 4th generation. Although the highest decrease in the growth of *Candida albicans* was observed in case of 40 and 50 ppm dettol, a sharp increase in the resistance was recorded at the successive generations at the same dettol concentrations. The 2nd generation showed 39.78% and 36.36% increase respectively compared with the 1st generation and reached 49.46% and 59.09% increase in the 4th generation compared with 1st generation. This trend showed that the higher the dettol concentration the higher the resistance, although the overall growth still weak compared with the growth of cultures with lower dettol concentration. This means that the growth is sharply decreased by increasing the dettol concentration. Fig (5) showed that the overall growth of *Candida albicans* in the first generation was lower than all the successive generations at all dettol concentrations and the sharp decrease at 40 and 50 ppm dettol was clearly indicated.

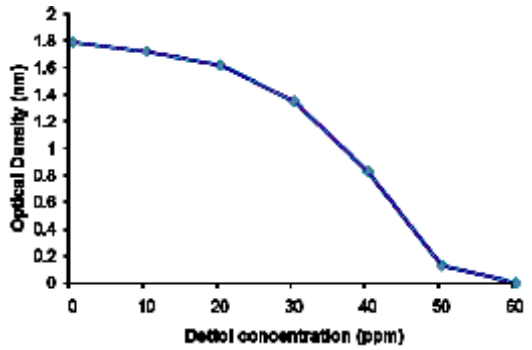

Fig. 1. The effect of different concentrations of dettol (ppm) on the growth (Optical Density (OD) at 550 nm) of *C. albicans*. Each value is the mean of three reading ± SE of the mean.

DISCUSSION

Effect of dettol on growth of *Candida albicans*

The result in the current study showed that the higher the dettol concentration the lower the growth of *Candida albicans*. No or low effect on *Candida albicans* of 10 ppm and 20 ppm dettol where 50 ppm cause 92.3% reduction. Complete inhibition was achieved at 100 ppm. Atayese *et al*., (2010) reported that the factory recommended dettol dose (5000 ppm) caused the complete inhibition of *Candida albicans* in 180 sec while the concentrate dettol without dilution (50000 ppm) led to the complete inhibition of *Candida albicans* in 90 sec. in the trend Olorode and Olorode and Okpokwasli (2012). Olorode *et al*., (2012) showed that 10% (5000 ppm) chloroxylenol reduced the growth of *Candida albicans* to zero after 20 minutes of exposure. However the above mentioned studies reported 50 fold higher concentration of dettol compared with the result of the current study that causes the complete inhibition.

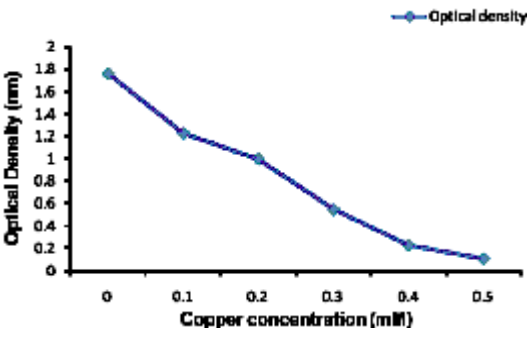

Fig. 2. Effect of different concentrations of copper chloride on the growth (Optical Density (OD) at 550 nm) of *Candida albicans*. Each value is the mean of three reading ± SE of the mean.

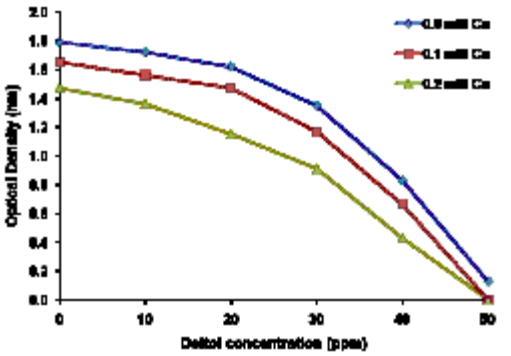

Fig. 3. Effect of different concentrations of dettol amended with 0.1 and 0.2 mM copper chloride on the optical density (O D) of *Candida albicans* (absorbance at 550 nm). Each value is the mean of three readings ± SE of the mean.

Effect of copper chloride on the growth of *Candida albicans*

Metal-microbe interactions have been the subject of considerable research attentions because of the biotechnological potential of microorganisms for metal removal or recovery, and toxicity for heavy metals towards microbial metabolism and growth (Poole and Gadd, 1989). The biocidal properties of copper have been known for centuries and have long been exploited to control or prevent the growth of a wide variety of microbial organisms (Borkow, 2009). Copper and some other metals toxicity is the basis of many fungicidal preparations of the control of pathogens and preservation of natural and man-made materials (Foye, 1977).

In the present study the increasing in copper concentrations cause a decrease in the growth of *Candida albicans*. The trend observed in this study showed that the higher the concentration of copper chloride the higher the percent reduction of the growth. At 0.1 mM the percent reduction was 30.11% where at 0.2 mM the percent reduction reached 43.18%. The maximum percent reduction (93.75) was at 0.5 mM. However, Weissman *et al* (2000) reported that *C. albicans* was able to grow up to 20 mM $CuSO_4$ in synthetic medium. This disagreement with the results in this study may due to the difference between the chloride ion and the sulfate ion in the copper salt used in both studies.

Resistance in four successive generations of *Candida albicans* to dettol

In the present study, *Candida albicans* showed an increase in the resistance to dettol in successive generations where the highest decrease in the growth was observed in case of 40 and 50 ppm dettol, however, a sharp increase in the resistance was recorded where the 2nd generation showed 39.78% and 36.36% increase respectively compared with the 1st generation and reached 49.46% and 59.09% increase respectively in the 4th generation compared with 1st generation. Le Chevalier *et al.* (1998) reported that resistance is inherent character of pathogens to chemical disinfectants and is not obtained or started as a result of disinfectants exposure but rather through specialized structures helping microorganisms' survival. Certain *Candida albicans* strains produce a biofilm from gelatinous material assist organisms attach to ecosystem surfaces and simultaneously protect them from exposure to disinfectants (Cornelis, 2008). Other pathogenic bacteria may also stick to this gelatinous material and use such protective nature of this biofilm (Hardie *et al.*, 2009)

Effect of addition of copper to dettol on the growth and germ tube formation of *Candida albicans*

A decrease in the absorbance of *Candida albicans* cultures as a result of adding copper to dettol was detected. The % reduction was increased from 3.9% due to 10ppm dettol and from 7.8% due to 0.1 mM copper to 12.8% due to addition of 0.1 mM copper to the 10ppm dettol. This increased reduction was observed in all treatments in this experiment. The maximum reduction percentage (100%) was achieved with the addition of 0.1 and 0.2 mM copper to 50ppm dettol.

The higher the stress over the organism the higher the number of germ tubes. This positive correlation is not only related to the number of germ tube but also related to the increasing number

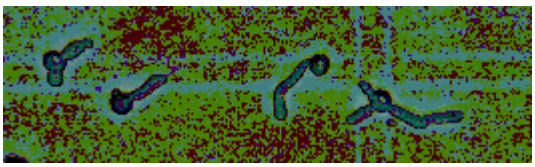

Fig. 4. Germ tube of *Candida albicans* formation after the addition of 20ppm dettol aided with 0.2 mM copper.

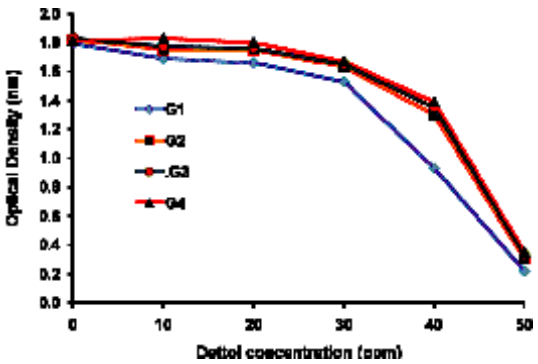

Fig. 5. Effect of different concentrations of dettol (ppm) on the culture optical density (O D) (absorbance at 550 nm) of four successive generations (G1, G2, G3 and G4) of *Candida albicans*. Each value is the mean of three readings ± SE of the mean.

of dead cells. So in fact the real effect of dettol and Copper is the acceleration of the growth profile toward the increasing number of cells and death of the cells in shorter time compared with the normal growth profile which will end with the higher cell number but in a longer time.

To the best of our knowledge the increasing lethality of dettol due to the addition of copper may be a novel practice. However, Olorode, (2012) reported that *Candida albicans* was more sensitive to Chlorxylenol alone compared with dettol that contain some additives to Chlorxylenol.

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