Journal of Kerbala University , Vol. 13 No.2 Scientific . 2015

Study the effects of different non-specific ototopical antifungal agents to treat Otomycosis

دراسة تأثير مضادات الفطريات الموضعية غير المحددة لعلاج التهاب الاذن الفطري

*Abbas Razzaq Abed \ Ph.D. Vet. Pharmacology \ dr_abas2008@yahoo.com

* Ibtisam Mohammed Hussein \ M.Sc. Bio-control \ ibtisammohmmed@yahoo.com

*Al-Furat Al-Awsat Technical University /Babil technical institute

Abstract

The current study used three different types of non-specific ototopical antifungal agents (3% H_2O_2, 1% Iodine and 2% Acetic acid) against common fungi (*Aspergillus niger* and *Candiada albicans*) which cause otomycosis in animals and human. All non-specific ototopical antifungal agents used in this study have antifungal activity against *Aspergills niger* but in different activity where he showed 2% Acetic acid decrease in redial growth zone of *Aspergillus niger* on SDA media compare with 3% H_2O_2 and 1% Iodine (9.40, 45.00 and 23.60 mm respectively) ,also 2% Acetic acid showed decrease in MIC value against *Aspergillus niger* compare with 3% H_2O_2, 1% Iodine (0.32, 0.80 and 20 mg/ml respectively) .Also noted the highest inhibition zone(17.33 mm) in 3% H_2O_2 against *Candida albicans* on SDA media , while no inhibition zones were seen in 1% Iodine and 2% Acetic acid, while the MIC value of 3% H_2O_2 against *Candida albicans* was lowest (0.16 mg/ml) compare with Iodine and Acetic acid (0.80 and 1.60 mg/ml respectively).

الخلاصة

استخدمت الدراسة الحاليه ثلاثة انواع من مضادات الفطريات الموضعية غير المحددة (3% H_2O_2, 1% Iodine and 2% Acetic acid) ضد الفطريات (*Aspergillus niger* and *Candiada albicans*) الشائعة في اصابة الاذن الفطرية في الحيوان و الانسان. جميع مضادات الفطريات الموضعية غير المحددة المستخدمة في هذه الدراسة تمتلك فعالية مضادة للفطريات ضد *Aspergillus niger* لكن بفعالية مختلفة حيث اظهر حامض الخليك 2% انخفاض في النمو الشعاعي الدائري لفطر *Aspergillus niger* في وسط اكار السابرويد السكري بالمقارنة مع 3% بيروكسيد الهيدروجين و 1% ايودين (9.40 و 45.00 و 23.60 ملم على التوالي) , كذلك اظهر 2% حامض الخليك قلة في قيمة التركيز المثبط الأدنى ضد *Aspergillus niger* بالمقارنة مع بيروكسيد الهيدروجين و الايودين (0.32 و 0.80 و 20 ملغم / مل على التوالي), كذلك لوحظ اعلى قطر تثبيطي(17.33 ملم) مضاد للفطريات في 3% بيروكسيد الهيدروجين ضد ***Candida albicans*** على وسط اكار السابرويد السكري , بينما لم يشاهد اي قطر تثبيطي ل1% ايودين و 2% حامض الخليك , بينما قيمة التركيز المثبط الأدنى لبيروكسيد الهيدروجين ضد ***Candida albicans*** كان اقل (0.16 ملغم/مل) مقارنة مع الايودين و حامض الخليك (0.80 و 1.60 ملغم/مل على التوالي) .

Introduction

Otomycosis is one of the common conditions encountered in a general otolaryngology clinic setting and it is an acute, subacute or chronic fungal infection of the pinna, external auditory meatus and the ear canal. Otomycosis as a true pathologic entity, with Candida and Aspergillus as the most common fungal species isolated (1). *Aspergillus niger* and *Candida albicans* are the most common causative agents of otomycosis (2)**.**

Predisposing factors of chronic infections otomycosis may be result from , use of oils, eardrops, , swimming ,fungal infection elsewhere in the body like dermatomycosis , immuno-compromised state, (3). Treatment options for otomycosis include elimination of predisposing factor, thorough canal cleaning and antifungal agents. Topical antifungals are specific (clotrimazole, miconazole, econazole, nystatin, tolnaftate, potassium sorbate), and non-specific (Acetic acid, alcohol, boric acid, m-cresyl acetate, and gention violet) (4).

The aim of this study include the comparative effect of different non-specific antifungal agents (3% H_2O_2 , 1% Iodine and 2% Acetic acid) on the most common mould (*Aspergillus niger*) and yeast (*Candida albicans*) which causes otomycosis in Human and animals.

Materials and Methods

Non-specific ototopical antifungal agents

Three types of most common non-specific ototopical antifungal agents were used in this study include 3% H_2O_2 (5), 1% Iodine (6) and 2% Acetic acid (7).

Stock solution preparation

Different concentrations of non-specific ototopical antifungal agents were prepared from original stock solution according to V1C1=V2C2 equation (8) where it 3% H_2O_2 was prepared from 50% H_2O_2 , while 1% Iodine was prepared from 10% Iodine and 2% Acetic acid was prepared from 98% Acetic acid. Distilled water used as diluents solvent to prepare different concentrations of non-specific ototopical antifungal agents.

Mycological isolates

In this study was used mostly fungi which causes otomycosis in human and animals (*A. niger* and *C. albicans*) (9,10), these isolates were taken from Bank of Microbiology which follow to Technical institute of Babil /Community health dept.

Spore counts of *Aspergillus niger*

The germination test protocol was adopted from (11). Briefly, after *A. niger* was cultured on the SDA medium at 27 ºC for 5 days, Conidia were collected and suspended in five ml of sterile normal saline. The spore concentration in the suspension was determined by using a haemocytometer method which include One drop of the suspension was added into hemocytometer chamber, spores were calculated under high power 40X of light microscope using the following equation(12) :-

$$\text{Spore number/ml} = \frac{Z \times 4 \times 10^6}{N}$$

Where:-Z= total number of counted spores (Spores number in 5 small square of RBCs count). N= total number of small squares (5 small square of RBCs count x 25 small square in each small square of RBCs count =80).

Final spore suspension must be obtained equal to 10^7 spore /ml.

Candida albicans cellular counts

C. albicans cells suspension was prepared by adding five ml of distilled water to fresh SDA media contains *C. albicans* (colonies aged 24 hour at 37^{o}C). Its turbidity was adjusted accordance to the absorbance of 0.08-0.10 at 625nm corresponding to 5 x 10^6 CFU/ml (13).

Antifungal sensitive test

1- *Candida albicans*

Agar disc diffusion method(14) was used to test for the inhibition activity of the different concentration of non-specific ototopical antifungal agents against *C. albicans.* The fungi were cultured on 20 ml SDA media in plastic petri-dishes. Inoculums of 0.1ml yeast cells suspension (cellular suspention 5 x 10^6 CFU/ml) were spread uniformly over this medium by using the spreader. Taken 50µl from each concentration of 3% H_2O_2, 1% Iodine and 2% Acetic acid and added into each disc (A five mm diameter disc was made from Whatman No.1 filter paper after that sterilization by autoclave 121ºC, 15 Ib for 15 minutes) (15) then dried by using electrical drier and put on the SDA medium and allowed to stand on the bench for one hour for proper diffusion. Number of petri-dish for each concentration repeated 5 times and each petri-dish contained about four discs. Inhibition activities of the concentration of non-specific ototopical antifungal agents were determined by measuring the zones inhibition formed around the discs in millimeter. The plates were observed for presence of zones of inhibition around the discs after 24 hours. Same previous steps were used again for distilled water which considered as control group.

2-*Aspergillus niger*

Antifungal activity of non-specific ototopical antifungal agents

According to V1C1=V2C2 equation, original non-specific ototopical antifungal agents (50% H_2O_2,10% Iodine and 98% Acetic acid) were mixed with SDA media after sterilization (Autoclave temperature 121ºC , For 15 mints at 15 Ibs) to obtain on 3% H_2O_2,1% Iodine and 2% Acetic acid then five mm diameter disc of *A.niger* mycelia were cut by sterilized cork borer from the periphery of 7 day old culture and transferred aseptically in the centre of SDA media contains different non-specific ototopical antifungal agents according to a pre-prepared concentrations. All petri plate including control and experimental were incubated at 28^{o}C for 7 days. After 7 days of incubation, observations were recorded and measurement of radial growth of *A. niger* (16).

Determination of minimum inhibitory concentration (MIC) of *Aspergillus niger* and *Candida albicans*

MIC of the effective non-specific ototopical antifungal agents were determined by tube dilution Method (17). , Ten test tubes with 8 ml of Sabouraud Dextrose Broth (SDB) in each were taken and autoclaved. To the first tube, 2 ml of the each concentration (50% H_2O_2,10% Iodine and 98% Acetic acid) was added and serial double fold dilution was done up to the 10 tube and from the 10 tube, 2 ml of the mixture was discarded. To each tube 100µl of inoculums (*C. albicans* suspention (5 x 10^6 CFU/ml) and ***A. niger*** (Spores 1x10^7 spore/ml) were added and mixed. The tubes were incubated for 48 hours at 37ºC for *C. albicans* and 5 days at 28ºC for ***A. niger*** . The least concentration of non-specific ototopical antifungal agents capable of inhibiting the fungal growth was considered MIC.

Aspegillus niger staining

A small portion of *A. niger* mycelium was cut by mycological loop (A small piece was taken from edge of zone growth of *A .niger* which grow on SDA media) and it place on the clean surface of class slide and mixed with one drop of Lactophenol-cotton blue dye . The mycelium was spread very well on the slide. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with X10 and X40 objective lenses respectively (18).

Statistical analysis

Statistical analysis of the experimental results were conducted according to Statistical Package for the Social Sciences (SPSS) version 13.00 where one way ANOVA was used to assess the significance of changes between experimental groups. The data were expressed as Mean ± Standard Errors (SE) and P-value<0.05 was considered statistically significance. LSD was carried out to test the significance levels among means of treatments (19).

Results and discussions

Effects of non-specific ototopical antifungal agents on the *Aspegillus niger.*

The effect of different non-specific ototopical antifungal agents which mixed with SDA media at a ratio 2% Acetic acid , 3% H_2O_2 and 1% Iodine showed a significant decrease in zone growth diameter of *A. niger* after 7 days of incubation at 28^{o}C in comparison with the SDA media which contains normal saline and distilled water. On the other hand the zone growth diameter of *A.niger* on SDA media which contains 2% Acetic acid showed a significant decreased in comparison with other SDA media which contains 3% H_2O_2 , 1% Iodine . Table (1) and figure (1).

Table (1):- Zone growth diameter of *A. niger* in the SDA media which contains different non-specific ototopical antifungal agents in comparison with normal saline and distilled water. The age of colonies 7 days at 28 ºC.

non-specific ototopical antifungal agent	Zone growth diameter (mm) M±SE
2% Acetic acid	9.40±0.24 A
3% H_2O_2	45.00±1.34 B
1% Iodine	23.60±1.28 C
Normal saline	76.60±0.67 D
Distilled water	77.40±0.92 D

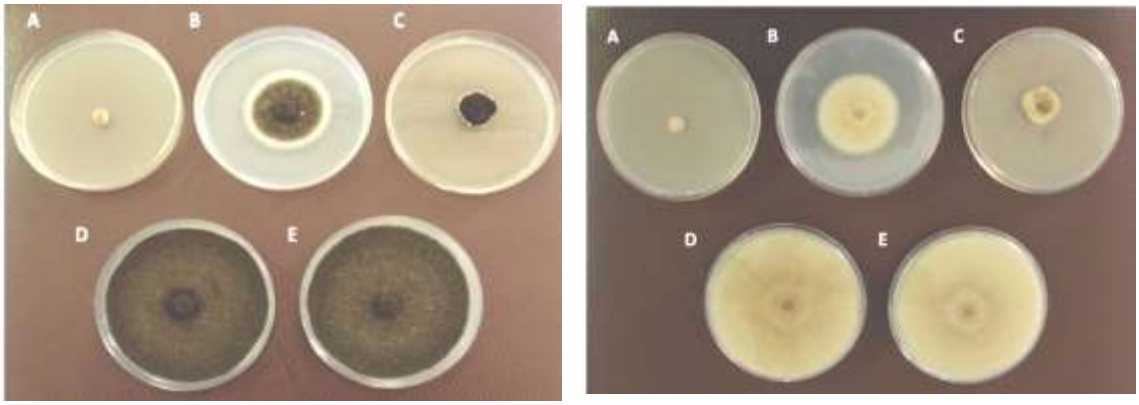
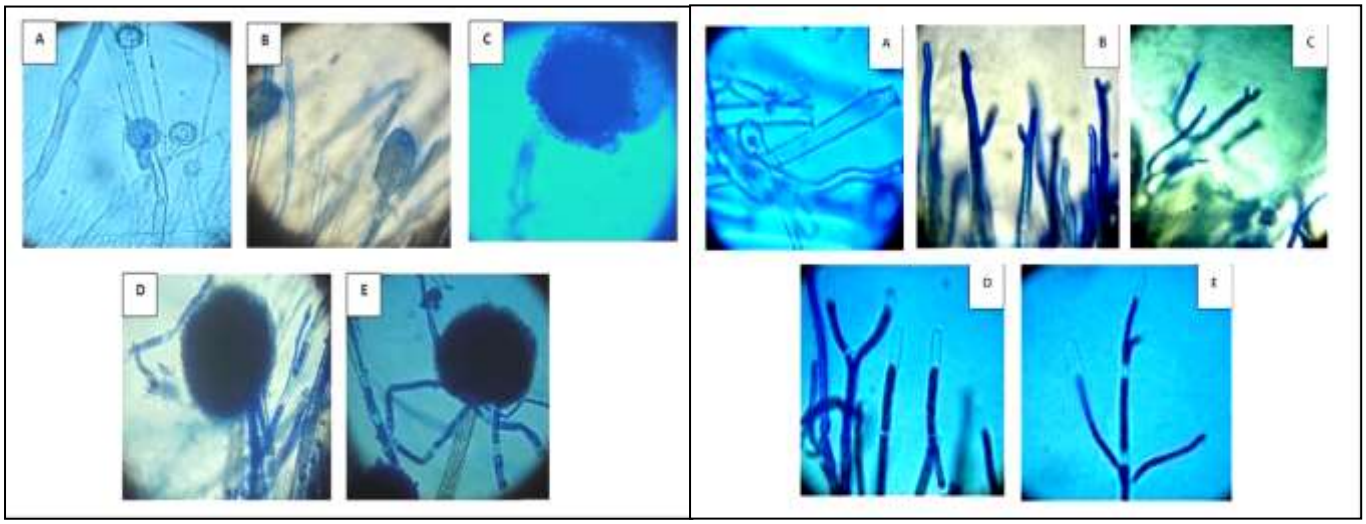


Figure (1):-Zone growth diameter of *A. niger* on SDA media which contains different concentration of non-specific ototopical antifungal agents (A= SDA media contains 2% Acetic acid, B= SDA media contains 3% H_2O_2, C= SDA media contains 1% Iodine, D= SDA media contain normal saline and E= SDA media contains distilled water. The age of colonies 7 days at 28 ºC. [Left side represent Front view of petri dish, while Right side Rear views of the Petri dish).

Microscopic appearance of *A. niger* cultivated on the SDA media which containing 2% Acetic acid and 3% H_2O_2 showed deformity in the sporangium structure (absence of Metulae , Phialides and Conidia) and growing tops of mycelia (growing tops in the SDA media which containing 2% Acetic acid appeared as end cuts, while growing tops in the SDA media which containing 3% H_2O_2 appeared a thick end with small branches, whereas *A. niger* cultivated on the SDA media which containing 1% Iodine showed weakness of conidiophores and growing tops of mycelia appeared a thick end with small branches , while sporangium structure nearly same as to the *A. niger* cultivated on the SDA media which containing normal saline and distilled water, , figure (2).



igure (2):-Microscopic appearance of *A .niger* on SDA media which contains different concentration of non-specific ototopical antifungal agents (A= SDA media contains 2% Acetic acid, B= SDA media contain 3% H_2O_2, C= SDA media contain 1% Iodine, D= SDA media contain normal saline and E= SDA media contain distilled water. The age of colonies 7 days at 28 ºC. [Left side represent *A. niger sporangium*, while Right side represent *A. niger* growing tops].

The lowest MIC value (mg/ml) of different non-specific ototopical antifungal agents against *A.niger* on SDB for 7 days at 28 ºC showed in Acetic acid in comparison with H_2O_2 and Iodine, table (2).

Table (2):-MIC value of different non-specific ototopical antifungal agents against *A. niger* for 7 days at 28 ºC.

Non-specific antifungal agent	MIC mg/ml
Acetic acid	0.32
Iodine	20
H_2O_2	0.8

All non-specific ototopical antifungal agents which used in this study have antifungal effects against *A. niger* and these effects depend on the mechanism of action of H_2O_2, Iodine and Acetic acid.

The results obtained in the current study noticed the antifungal activity of 2% Acetic acid against *A. niger* was more potent than 3% H_2O_2 and 1% Iodine and these results may be resulted from several reasons.

First cause, the antifungal activity of 3% Acetic acid may be resulted from protein autolysis of *A. niger* mycelia, inhibited sporulation , breakdown of ribosomal RNA occurred and decreased the dry weight , contents of glycogen-like polysaccharide, soluble protein . Also Acetic acid caused breakdown of macromolecules in mycelia (20).

Second cause which related with acidic media which resulted from mixture of 2% Acetic acid with SDA media may resulted environment inappropriate for growth of *A. niger* (21). In addition the antimicrobial activity of Acetic acid in aqueous solution is pH dependent, with the maximum effect occurring at low pH, thus favoring the undissociated state of the acid (22). Because they are uncharged, undissociated acid molecules are lipophilic and will penetrate plasma membranes and

thus enter cells. Theoretically, the higher-pH environment of the cell cytosol in *A. niger* (23) promotes the rapid dissociation of acid molecules into charged protons and anions, which cannot subsequently diffuse back across the plasma membrane. Intracellular acidification of the cell cytosol resulting from the accumulation of protons inhibits key metabolic activities involved in glycolysis (24) and hence inhibits ATP yields. A reduction in intracellular pH (pH_{int}) and thus in the proton motive force (Δp) may also lead to reduced cellular uptake of amino acids (25)**.**

Hydrogen peroxide is known to be a very powerful oxidizing agent that is in general effective against a wide spectrum of microorganisms including bacteria, yeasts, molds, viruses and spore-forming organisms (25). This antimicrobial effect may be resulted from cytotoxic oxidizing species such as hydroxyl radicals (26). In this study the antifungal effect of hydrogen peroxide (On SDA media or MIC) was lower than Acetic acid this may be resulted from low hydrogen peroxide concentration used or this fungus need hydrogen peroxide concentration higher than 3% to prevent growth this fungus.

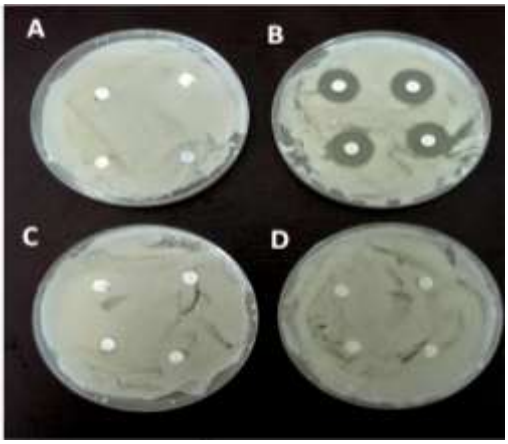
Iodine is one of the halogen group compounds and its broad spectrum compounds that are considered low toxicity, low cost and easy to use (27)**.** Iodines act by denaturing proteins to interfere with the enzymatic systems of microorganisms (21). Antifungal effects of 1% Iodine was lower effects than Acetic acid and hydrogen peroxide this results may resulted from low Iodine concentration used or this fungus need Iodine concentration higher than 1% to prevent growth this fungus.

Effects of non-specific ototopical antifungal agents on the *Candida albicans*.

The present study showed a significant increase ($P<0.05$) in the zone inhibition of 3% H_2O_2 against *C. albicans* , while no zone inhibition showed in 2% Acetic acid , 1% Iodine and distilled water table (3) and figure (3).

Table (3):-Zone inhibition diameter of different non-specific ototopical antifungal agents against *C. albicans* in the SDA media in comparison with distilled water. The age of colonies 24 hours at 37ºC.

Non-specific antifungal agent	Diameter of zone of inhibition (mm) M±SE
2% Acetic acid	0.00±0.00 A
1% Iodine	0.00±0.00 A
3% H_2O_2	17.33±0.57 B
Distilled water	0.00±0.00 A



figure(3):-Inhibition Zone diameter (disc-diffusion method) of non-specific ototopical antifungal agents against *C. albicans* in the SDA media for 24 hours at 37 °C in comparison with distilled water A= 2% Acetic acid, B= 3% H_2O_2 C=1% Iodine , C= 1% Iodine, D= distilled water.

The lowest MIC value (mg/ml) of non-spesific ototopical antifungal agents against *C.albicans* on SDB for 48hour at 37ºC showed in H_2O_2 in comparison with Acetic acid and Iodine, table (4).

Table (4):-MIC values of non-specific ototopical antifungal agents concentration against *C.albicans* for 24 hours at 37 ºC.

Non-specific antifungal agent	MIC mg/ml
Acetic acid	1.60
Iodine	0.80
H_2O_2	0.16

In the current study the antifungal activity of 3% H_2O_2 against *C. albicans* was more potent than 1% Iodine and 2% Acetic acid and this may be result from several causes, first cause *Candida* cant become resistant to Hydrogen Peroxide, because it can't live in the presence of oxygen (28).

Second caused H_2O_2 functions as an oxidizing agent by producing free radicals that react with lipids, proteins and nucleic acids to affect cellular constituents non-specifically (29).

The current study showed that the antifungal activity of Acetic acid affect on transport of necessary nutrient through cytoplasmic membrane which was destroyed due to increasing acidity (30). Also the toxic effect of acetic has ability by penetration of weak acids inside the microbe stopping the nucleic acid and protein synthesis. The antifungal activity of Acetic acid refers to the presence of potassium hydroxide which separated from apple Acetic acid which prevents the absorption of water by microorganisms leading to stopping the growth (31)**.**

Iodine has antiseptic effects by replaced by substances known as iodophores that contain an Iodine molecule linked to a large molecular-weight organic compound (32). Release of free Iodine from the iodophore contributes to its biocidal activity. The liberated Iodine is believed to penetrate the outer wall of microbes, and kill the cells by disrupting protein structure and nucleic acid synthesis (33).

The verity of antifungal effects of Iodine and Acetic acid against *C. albicans* in comparison with H_2O_2 may be resulted from lowest concentration of Iodine and Acetic acid were used in the treatment of *C. albicans* .

Conclusions

1- All materials used in this study showed the effect on the growth of fungi , but to varying degrees.
2- Antifungal effects of Acetic acid against *A. niger* was more potent than Iodine and H_2O_2.
3- An antifungal effect of H_2O_2 against *C. albicans* was more potent than Iodine and Acetic acid.

Recommendation

1. Molecular study of Iodine, Acetic acid and H_2O_2 effects on the cellular structure of *A. niger* and *C. albicans*.
2. A study about the possibility of drug combination (synergistic effect) work between Acetic acid and Iodine to get on the common anti-fungal medication using to molds and yeasts that infect the ear.

References

1-Tang .H ; Jeffrey , T. ; Donald, Y. and Newton, J. ;Coker, MD and Houston, T. (2006). Otomycosis: Clinical features and treatment implications. American Academy of Otolaryngology–Head and Neck Surgery Foundation.135 :787-791.

2-Latha , R. ; Sasikala , R , and Muruganandam, N. (2010). Chronic Otomycosis Due to *Malassezia* Spp. J Glob Infect Dis., 2(2): 189-190.

3-Ashish, K.(2005). Fungal Spectrum in Otomycosis Patients. JK science. 7 :3.

4-Chalabi, EY. and Ahmed, TS .(2010). The role of various out patient's aural toileting procedures in the treatment of otomycosis. J. Zankoy Sulaimani.13:39-48.

5-Karen, K.(2012). Managing otitis externa. S. Afr. Pharm. J., 79(8):17-22.

6-Spandow, O; Anniko, M and Moller, AR.(1988). The round window as access route for agents injurious to the inner ear. Am J Otolaryngology, (6):327-335.

7-Ahmad, Y.; Mohammad, A. ; Rohullah ,D. ; Ali, Z. ; Mahmoudabadi ; Fariba, R. ; Hossein, H. and Ahmad, K.(2010). Evaluating the effect of a mixture of alcohol and acetic acid for otomycosis therapy . Jundishapur Journal of Microbiology, 3(2): 66-70.

8-Mary, A. H. ; Robert L. K. and John, C. P. 2005. Mathematics Exercises in Biotechnology. National Science Foundation .NSF Award #: DUE 0003065.

9- Jadhav, V.J.; Pal, M. and Mishra, G.S. (2003). Etiological significance of *Candida albicans* in otitis externa. Mycopathologia, 156: 313-315.

10-Pankti, P. ; Jayshree. P. ; Dipt,i P.; Sanjay, R. and Parul, S. (2013).Analysis of various fungal agents in clinically suspected cases of otomycosis. Indian Journal of Basic and Applied Medical Research. 8(l-2):865-869.

11-Nesci, A.; Rodriguez, M. and Etcheverry, M. (2003) Control of Aspergillus growth and aflatoxin production using antioxidants at different conditions of water activity and pH. J. Appl. Microbiol., 95,279-287.

12-Faraj , M.K.(1990). Regulation of mycotoxin formation in Zea mays. Ph.D. dissertation, Department of Bioscience and Biotechnology University of Strathclyde , Glascow. U.K.

13-Ricardo, D. and Edeltrudes, O. (2013). Anti-Candida Activity and Chemical Composition of *Cinnamomum zeylanicum* Blume Essential Oil .Braz. Arch. Biol. Technol., 56 (5):749-755.

14-Maidment, C. ;Dyson, A. and Haysom, I. (2006). A study into the antimicrobial effects of cloves (*Syzgium aromaticum*) and Cinnamon (*Cinnamomum zeylanicum*) using disc-diffusion assay. Nutr. Food Sci., 36 (4): 225-230.

15-Zohreh, D.; Mohammad, A.; Mohammad, A.; Maryam, A.; Farnaz, F. ; Pegah, M.; Mozafari, F. and Mojarad, Hi. (2011) .*In Vitro* Comparison of Inhibitory Activity of 10 Plant Extracts Against *Candida Albicans.* Australian J. of Basic and Applied Sci., 5(5): 930-935.

16-Mohit, K. ; Mohammed, F.; Satyapal, S.; Anwar, S. and Ashok, K. B.(2013). Antifungal activity of the Eucalyptus australe important medicinal plant. International Journal of Engineering Science Invention. 2(1):30-27.

17-Cruickshank, R.; Duguid, J. P. and Marmion, B.P. (1975). Tests for sensitivity of antimicrobial agents. Med. Microbiol., 190-208.

18- James, G. C. and Natalie, S. (2001). Microbiology. A laboratory Manual (ed.). 211-223.

19-Joda, M. (2008).The progressive statistical analysis by using SPSS. (1st ed.) Churchill livingstone .Edinburgh .

20-Koki, T.; Kazuo, M.; Hiroyuki, T. and Kazutami, W.(1977). Effect of Acetic acid on growth of *Aspergillus niger*. Journ. Fac. Sci., 10 (4): 189-198.

21-Maris, P.(1995).Modes of action of disinfectants. Rev. sci. tech. Off. int. Epiz., 1995:14(1):47-55.

22-Piper, P. C. ; Ortiz Calderon, K. ; Hatzixanthis, and Mollapour, M. (2001). Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147:2635-2642.

23-Hesse, S. ; Ruijter, C. and Visser, J. (2002). Intracellular pH homeostasis in the filamentous fungus *Aspergillus niger*. Eur. J. Biochem. , 269:3485-3494.

24- Krebs, H. A.; Wiggins, M. ; Stubbs, A. and Bedoya. F. (1983). Studies on the mechanism of the anti-fungal action of benzoate. Biochem. J., 214:657-663

25-Cords, BR. and Dychdala, GR. (1993).Sanitizers: halogens, surface-active agents and peroxides. In: P M Davidson and A L Branen, eds. Antimicrobials in foods, second edition. New York, Marcel Dekker, Inc., 469-537.

26-Juven, B. and Pierson, MD. (1996). Antibacterial effects of hydrogen peroxide and methods for its detection and quantitation. J. Food Prot, 59(11):1233-1241.

27-Jeffrey, DJ.(1995). Chemicals used as disinfectants: Active ingredients and enhancing additives. Rev. sci. tech. Off. int. Epiz., 14(1):57-74.

28-William, C. D.(2003).Hydrogen peroxide medical miracle . North America Rhino Publishing, S.A.

29-Sleigh, JW.; Linter, SP.(1985). Hazards of hydrogen peroxide. Br. Med. J. (Clin Res Ed) ., 291(6510): 1706.

30-Bangwart, GJ. (1989). Basic food microbiology, 2nd ed. New York: van nostrand rein fold, 78: 117-125.

31-Matousek, L. and Cambel, K.(2002). The effect of detergents on skin pH and it is consequences. Clin. Dermatol., 14: 147-58.

32-Kumar, JK. and Kumar, H. and Reddy,C.(2009). Application of broad spectrum antiseptic povidone Iodine as powerful action: A review. Journal of Pharmaceutical Science and Technology.1 (2):48-58.

33-McDonnell, G. and Russell, AD.(1999). Antiseptics and Disinfectants :Activity, Action, and Resistance. Clinical Microbiology Review, 12 (1):147-179.