Anticandida and immunomodulating activity of Apple cider vinegar; its use in treating denture stomatitis and as a root canal irrigate.

Gulafsha Khan, Mrs.Jyoti Mantri.
Student, Proffesor
Department of Microbiology,
Sophia College(Autonomous),Affiliated to the University of Mumbai,Mumbai,India.

I. Abstract: *Antibiotic resistance is rising to dangerously high levels in all parts of the world, making alternative antimicrobials essential. The high prevalence of oral candidiasis and the restricted number of antifungal agents available to control it, justifies the development of new therapies for use in daily clinical practice.The medical impact of C.albicans typically depends on its ability to form virulent factors and biofilms, which attach to surfaces, such as tissues and implanted medical devices and also dentures.These biofilms serve as drug-resistant reservoirs of cells that can multiply and cause denture stomatitis and subsequent bloodstream infections. Chlorhexidine gluconate is a widely used potent oral antiseptic that has side effects such as toxicity, burning sensation, teeth staining, disagreeable smell, and taste. This study compares activity of apple cider vinegar withChlorhexidine gluconate, in combating denture stomatitis caused by Candida albicans.Macrodilution technique was used to determine the minimum inhibitory concentration(MIC) and minimum fungicidal concentration (MFC) of apple cider vinegar and chlorhexidine. Apple cider vinegar showed MIC/MFC of 2500µg/ml whereas chlorhexidine showed MIC/MFC of 7.8µg/ml.The effect of ACV(test),CHX(standard) and distilled water(control) on inhibition of biofilm formation by Candida albicans using acrylic resin was studied by comparing the number of colony-forming units (cfu/ml) of the adhered microorganisms.The mean value of cfu/ml in the control group (distilled water), standard group (Chlorhexidine)and ACV(test) were 3×10^5,$8\times10^2$and 5×10^4respectively.This study further aims to assess the effect of ACV on the virulence factors, germ tube of C.albicans and phagocytic activity of monocytes that are the key effectors of innate immunity using flow cytometer.*

Key words: Apple Cider Vinegar (ACV), Denture stomatitis, C.albicans, Root canal failure.

II. Introduction:

In spite of all advances in dentistry, complete dentures are still essential for oral rehabilitation of edentulous dental arches. However, this type of prosthesis, which is basically confectioned with thermally activated acrylic resin, constitutes a favorable environment for the colonization and proliferation of *Candida* genus yeasts, since these microorganisms have the ability to strongly adhere to polymethylmethacrylate, which constitutes theacrylic resin. This phenomenon, in association with factors such as trauma, diet, poor hygiene or predisposing systemic conditions (xerostomia, hormonal alterations and immunosuppression caused by diabetes *mellitus* or HIV infection), may lead complete denture users to develop a condition known as denture stomatitis. Denture stomatitis is clinically characterized by a discrete focal inflammation on the palatum, which can evolve to an intense erythema in all area covered by the denture and, in some cases, to papillary hyperplasia [2]It has multifactorial etiology, but is especially associated with *Candida albicans*.Candida albicans is one of the most important fungal colonizers on the skin and mucosal surfaces of the body such as genitourinary tract, oral cavity, and gastrointestinal tract. The pathogenicity of *Candida albicans* is attributed to certain virulence factors, such as the ability to evade host defences, adherence, biofilm formation (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin.[3].The treatment of lesions associated with

denture stomatitis, encompasses denture andoral cavity hygiene instructions, removal of the irritant factor(prosthetic device), use of antifungal agents, and acquisition of anew denture.Some synthetic products that are used for the treatment of candidiasis are nystatin, miconazole, amphotericin B, fluconazole, itraconazole, and ketoconazole.Chlorhexidine is a potent antiseptic, which is widely used for chemical plaque control in the oral cavity and as an irrigating solution. Although chlorhexidine presents excellent antimicrobial properties such as low-concentration efficiency, minimal perception by the gastrointestinal tract, capacity to reduce biofilm formation and disorganize pre-formed biofilm, it also presents some side effects such as toxicity, burning sensation, teeth staining, disagreeable smell, and taste. [4]The increased number and use of conventional antifungals for the treatment of systemic and superficial infections has resulted in the selection of resistant strains in patients at risk for disseminated infections by *Candida*, particularly those individuals with severe and immunosuppressive diseases.[5].In spite of the use of chlorhexidine gluconate and sodium hypochlorite as thetwo most popular root canal irrigates a majority of root canal failures are caused by *C.albicans*.All of these facts justify the need for new therapiesthat could minimize the undesirable consequences of the conventional agents. Among the new alternatives under study for the treatment of denture stomatitis; maleic acid has been addressed as an important element contained in apple cider vinegar, with bactericidal and fungicidal activity.[6]The therapeutic effects of ACV can be attributed to the bioactive constituents of the organic acids generated in ACV production, including acetic, citric, formic, lactic, malic, which have antimicrobial, antioxidative, antidiabetic, antitumor, antiobesity, antihypertensive, and cholesterol-lowering properties. ACV consists of acetic acid, flavonoids such as gallic acid, tyrosol catechin, epicatechin, benzoic acid, vaninilin, caftaric acid, coutaric acid, caffeicacid, acid and ferrulic acid. These constituents are responsible for immune defence and oxidativeresponses. The immune-modulatory activity of ACV is due to its ability to downregulate cytokine expression and upregulate mononuclear phagocytic function. This anti-inflammatory potential is attributed to the apple polyphenol content.

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Therefore the main objective of the present study was to evaluate the antifungal activity of apple cider vinegar against *C.albicans*(main etiology for causing denture stomatitis and root canal failure) and compare it with Chlorhexidine gluconate, which is a conventional antifungal agent used for treating denture stomatitis and for root canal irrigation.

III. Materials and Methods:

1. Anti-Candida activity of ACV and Chlorhexidine gluconate by agar well diffusion method:

Candida albicans suspension was prepared by inoculating 2-3 pure colonies of Candida in St. Sabourauds broth and was incubated on shaker at 37º C for 24 hours. O.D of the broth was adjusted to 0.1 using St.Saline and0.1ml of the culture suspension was bulk seeded in 15ml of Mueller Hinton agar in a petridish. Subsequently, wells were punched into the agar medium and filled with 100 µl (40,000µg/ml) of the test solution(ACV) and 100 µl of Standard solution (1000 µg/ml Chlorhexidine gluconate). The plates were then incubated in the upright position at 37° for 24 h. After incubation, the diameters of the growth inhibition zones were measured in mm.

2. Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration(MFC) of apple cider vinegar containing 4% maleic acid, and Chlorhexidine (1%):

MIC was determined by the macrodilution technique. A total of 2 ml Sabouraud's Dextrose Broth (SDB) distributed in each tube, followed by 2 ml of test substance (apple cider vinegar or chlorhexidine gluconate at initial concentrations of 40,000 µg/ml and 1000 µg/ml, respectively). An aliquot of 2 ml was collected from the first tube and then dispensed into the following one, in order to proceed with a 2-fold serial dilution. Approximately 0.1 of inoculum were dispensed into each tubeexcept the negative control tube(media sterility control).The tubes were incubated at 37°C for 24 hours.MIC was considered as the lowest concentration able to inhibit visible yeast growth.

MFC is the minimum concentration of an antifungal agent that results in bacterial death.
Inorder to confirm the fungicidal potential of apple cider vinegar, Minimum Fungicidal concentration(MFC) was determined by streaking on a St. Sabouraud's agar plate from the MIC tube as well as from the tubes close to the Minimum inhibitory concentration.

3. Germ tube inhibition assay:

Candida pathogenicity is facilitated by a number of virulence factors, the most important of which is the ability to grow in a variety of morphological forms and at least, four kinds of forms, that is, yeast-like, hyphae, pseudohyphae, and chlamydospores have been well documented .Among these morphological forms, the yeast-hyphae switch has been extensively studied due to its correlation with pathogenicity.

The assay was performed by adding 1ml test and standard solution and 0.5ml fresh serum in 2ml St.Sabouraud's Dextrose broth. 0.1ml culture was added and incubatedat 37˚ C for 1 hour. In the control tube only media, serum and the inoculum was added. Inorder to visualize and compare the length of the hyphae in the presence of Apple cider vinegar compared to the Control tube without the test and standard solution, a drop of the solution was transferred on a glass slide, covered with a coverslip and observed under 10x and 45X at intervals.

4. Phospholipase inhibition assay:

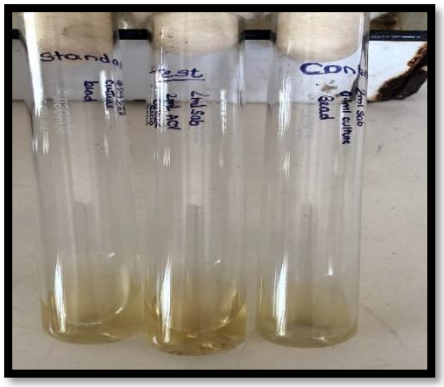
The extracellular phospholipases of *C. albicans* catalyze the hydrolysis of phospholipids which are major components of all cell membranes and therefore have a significant role in the pathogenesis of infections and invasion to mucosal epithelia.
0.1ml of *C.albicans* suspension was added to 0.5ml of ACV for 30, 60, 90,and 180 minutes and was spot inoculated at equidistant points on St.egg yolk agar medium. The plates were incubated at 37˚C for 24 hours. Zone of precipitation were measured after incubation.

5. Proteinase inhibition assay:

Proteinases of C.albicans are known to modify target proteins and ligands which lead to conformational changes allowing better adhesion of the fungus.
The proteinase inhibition assay was carried out as follows:
0.1ml of *C.albicans* suspension was added to 0.5ml of ACV for 30, 60, 90, and 180 minutes and was spot inoculated at equidistant points on St.Skim milk agar medium. The plates were incubated at 37˚C for 24 hours. Zone of precipitation were measured after incubation.

6. Test for inhibition of biofilm formation i.e. adherence to acrylic resin surface:

Biofilm formation by Candida albicans play an important role in pathogenesis because of their increased resistance to antifungal therapy and the ability of cells within biofilms to withstand host immune defenses.Also, biofilm formation on medical devices can negatively impact the host by causing the failure of the device and by serving as a reservoir or source for future continuing infections.
Five 12 mm^2 sterile acrylic resin specimens were obtained for each set and the inhibition of adherence was performed as follows:
Five acrylic resin specimens, 2 mL of Sabouraud's broth and 0.1 mL of *Candida albicans* standardized suspension were added to each tube. 1ml of the test solution at its sublethal concentration (ACV) and 1ml of standard solution (chlorhexidine) were added to the test and standard tubes respectively. Control tube contained only acrylic specimens, medium and the culture suspension. The tubes were incubated at 37˚C for 24 hours. After incubation, 1 specimen from each tube were washed with 1 ml saline and vortexed in 50 ml saline for half an hour in order to disperse the adhered cells, followed by serial dilution and plating on St. Sabouraud's agar plates. The plates were incubated for 24 hours and the number of colony forming units per specimen was determined. A graph of mean values of cfu/ml of *Candida albicans*per group (control, test and standard) was plotted.

7. Determination of phagocytic capacity of monocytes by Flow Cytometry:(proposed work)

ACV can decrease induced inflammatory cytokine release during mononuclear leukocyte infection and increases monocyte phagocytic capacity, which is significant as microbial phagocytosis is a key effector function of innate immunity. In order to investigate whether the phagocytic capacity of monocytes increases in the presence of ACV, the following procedure will be used:

i. Human mononuclear cell isolation from whole peripheral blood:

- Preparation of the sample:

To a 10ml centrifuge tube add 2 ml of defibrinated-or anticoagulant treated blood and an equal volume of balanced salt solution (final volume=4ml). Mix the blood and buffer by inverting the tube several times.

ii. Isolation of mononuclear cells:

1) Add Ficoll-hypaque solution (3ml) to a centrifuge tube.
2) Carefully layer the diluted blood sample (4ml) onto the ficoll-hypaque solution.
3) Centrifuge at 3000 rpm for 30 mins.
4) Draw off the upper layer containing plasma and platelets using a sterile pipette, leaving the mononuclear cell layer undisturbed at the interface.
5) Transfer the layer of mononuclear cells to a sterile centrifuge tube using a st.pipette.
6) Culture the freshly isolated monocytes at 4×10^5 cells/mL over a period of two days
7) Test: Incubate with *C.albicans*suspension (4×10^6 CFU/ml) for 4 h at 37 °C and 5%CO2 with varying concentrations of apple cider vinegar.
8) Control 1: Incubate with only *C.albicans* suspension.
9) Control 2: resting unstimulated monocytes.
10) Scrape the Cells and replenish in ice cold PBS containing 1 mM EDTA, wash. Fix the resultant pellets in 400 µL of 4% paraformaldehyde.
11) Analyze using a FACS Calibur flow cytometer. Analyze the changes in regional gated profiles and measure the % shift in side scatter.

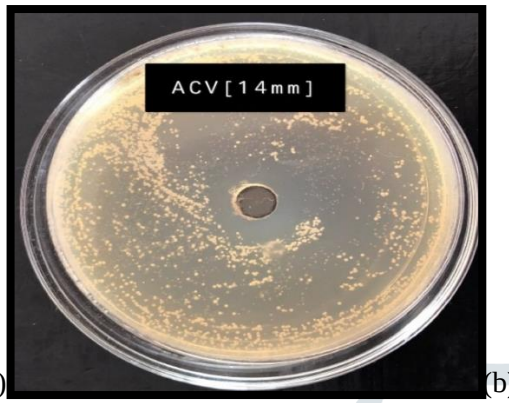
IV. Results.

1. Anti-Candida activity of ACV and Chlorhexidine gluconate by agar well diffusion method:

The antifungal activities of ACV and CHX tested against *C.albicans* by the agar disc diffusion method are displayed in Table 1.

Table:1

Solution	Zone of inhibition in mm
Apple cider vinegar (Test)	14
Chlorhexidine gluconate (Standard)	18

(a) 

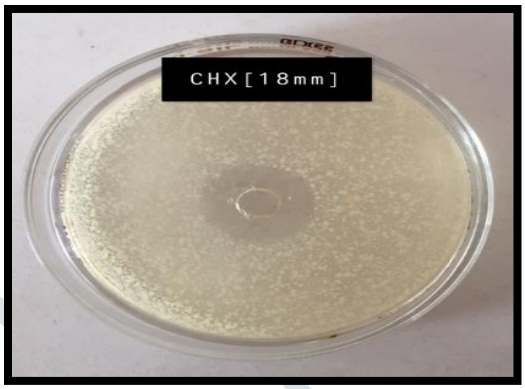
(b) 

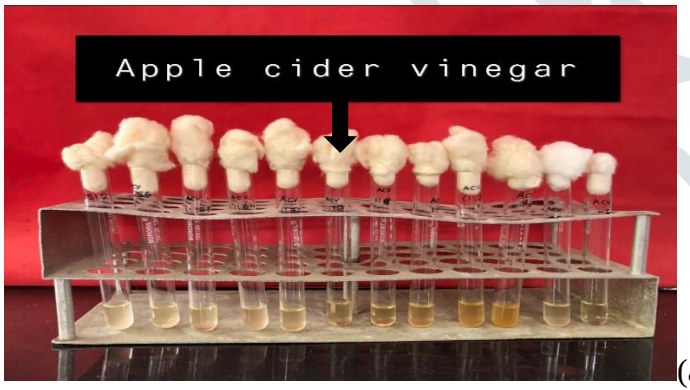
Figure 1: Screening of antifungal activity of (a) apple cider vinegar and (b) chlorhexidine gluconate against C.albicans by agar well assay

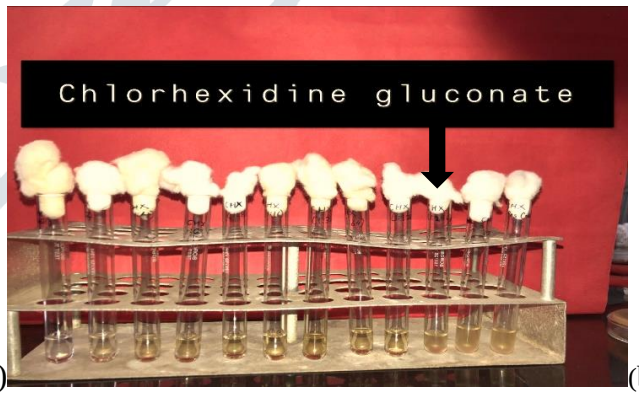
2. Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration(MFC) of apple cider vinegar containing 4% maleic acid, and Chlorhexidine (1%):

   MIC of apple cider vinegar was found to be 2500 μg/ml, which corresponds to 0.25% maleic acid, whereas MIC of chlorhexidine gluconate was 7.8 µg/ml. Photographs of the MIC assay are shown in Table 2.

Table:2

Solution	MIC(µg/ml)
Apple cider vinegar (Test)	2500
Chlorhexidine gluconate (Standard)	7.8



(a) 

(b)

Figure 2: photograph of MIC assay of (a) Apple cider vinegar and (b) chlorhexidine gluconate.

Minimum fungicidal concentrationwas determined by streaking on a St. Sabouraud's agar plate from the MIC tube as well as from the tubes close to the Minimum inhibitory concentration.MFC values were found to be same as MIC for both ACV and CHX.

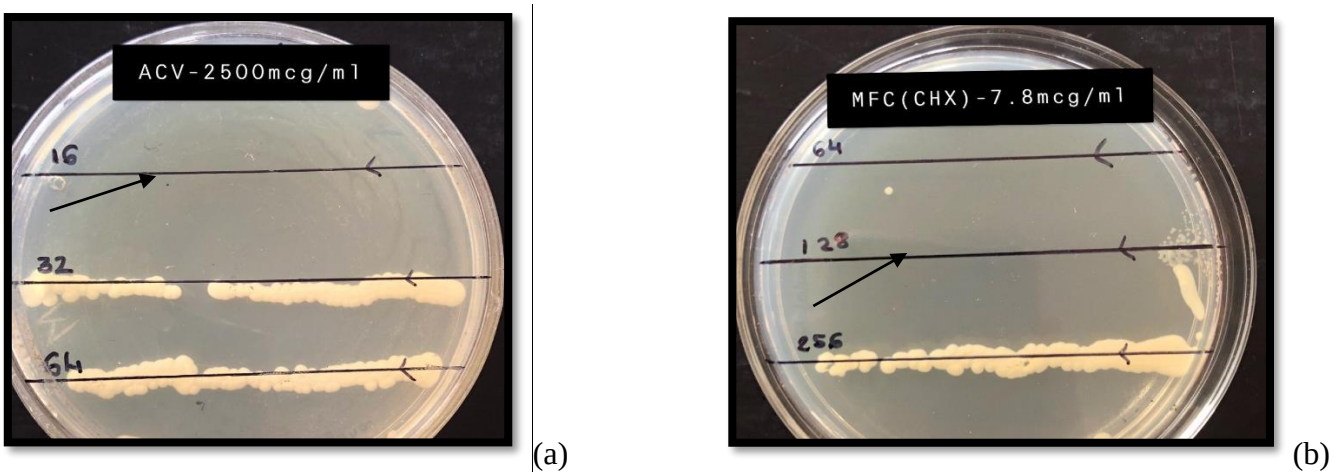


Figure 3: photographs of MFC assay.

3. Germ tube inhibition assay:

Germ tube inhibitory activity of apple cider vinegar and Chlorhexidine gluconate were screened. *C. albicans* culture was inoculated in the serum and incubated for 3 h at 37°C. Germ tube formation was observed under the microscope, at intervals of 30 mins. Control tubes were maintained similarly without test solutions and were observed for comparison of germ tube formation.It was observed that chlorhexidine gluconate could completely inhibit germ tube formation in *C. albicans* until the end of incubation period(figure 4) Apple cider vinegaralso showed germ tube inhibitory activity on *C. albicans* in the first hour of incubation and at the end of incubation period (90mins), only few budding cells were observed as compared to the control in which long hyphal (filamentous) extension arising laterally from the cells were observed(figure 5 and 6)

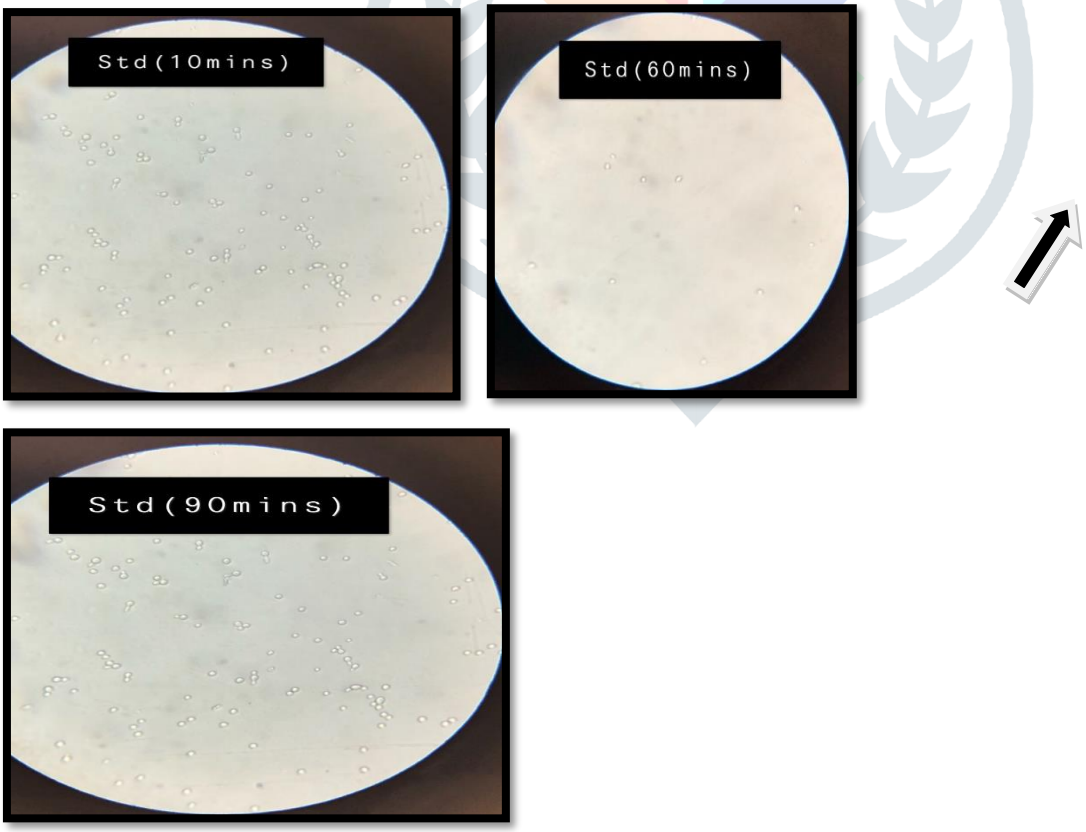


Figure 4:Screening of germ tube inhibitory activity of CHX (standard)in *Candida albicans.*

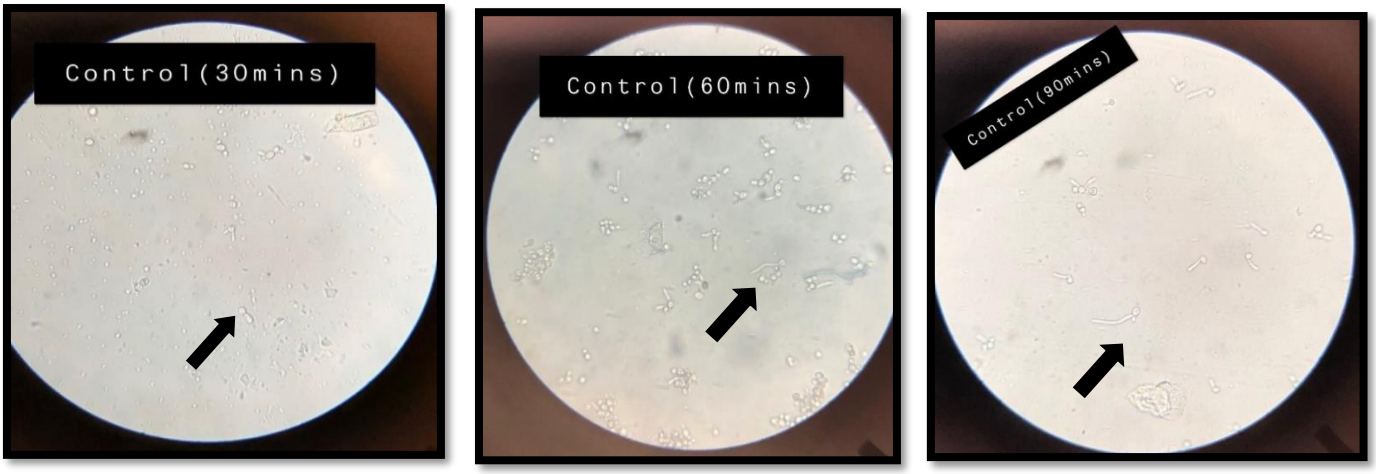


Figure 5: Screening of control with no test solutions to compare germ tube formation.

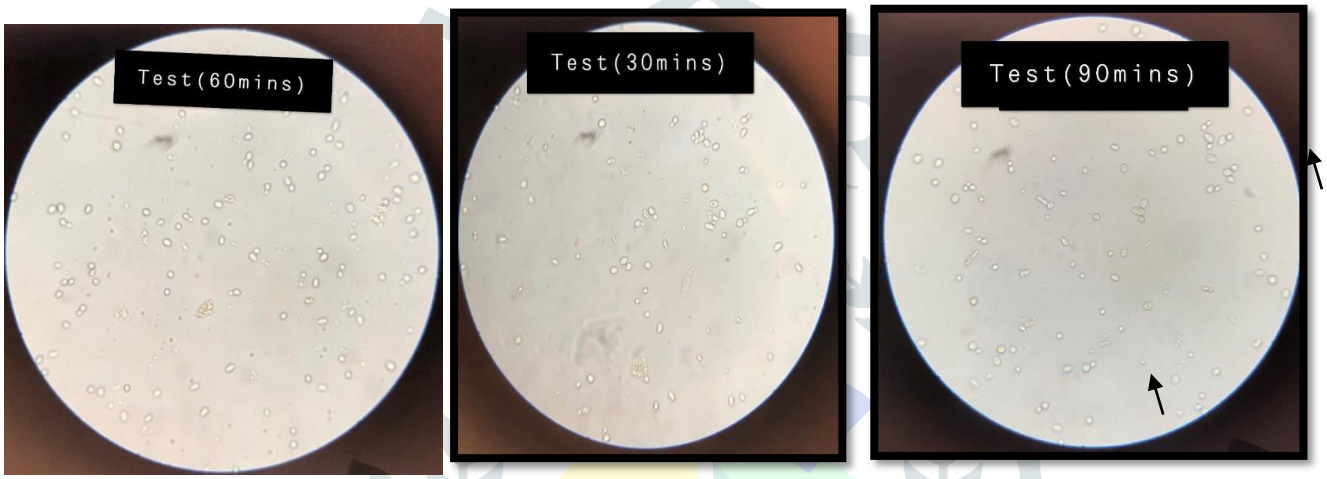
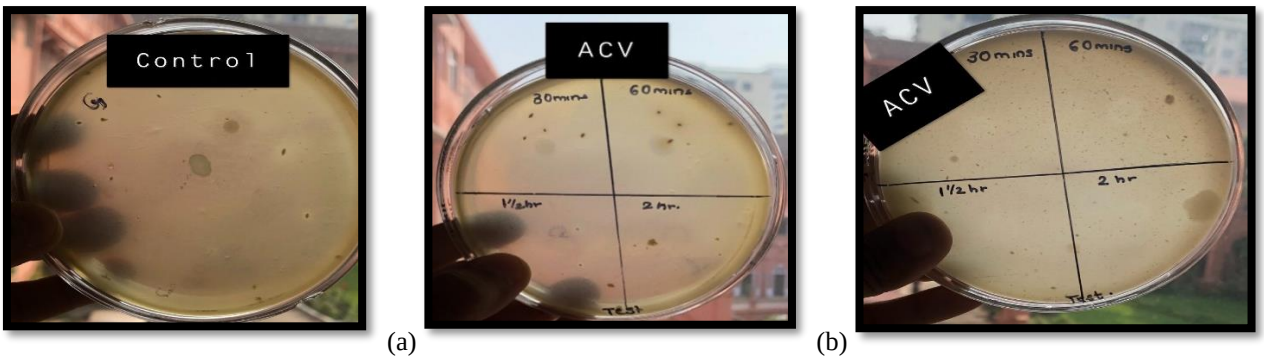


Figure 6:Screening of germ tube inhibitory activity of ACV (test)in *Candida albicans.*

4. Proteinaseand Phopholipase inhibition assay:

The proteinase and phospholipase inhibitory activity of apple cider vinegar was detected using 10% milk agar and egg yolk agar respectively. *C.albicans* suspension and ACV was incubated for 30, 60, 90, and 180 minutes and was spot inoculated at equidistant points on the medium. The zone diameter gradually decreased and the colony that was spot inoculated after 2hours showed no clearance indicating that ACV had completely inhibited the virulent enzymes.(Figure:7)



(a) (b) (c)

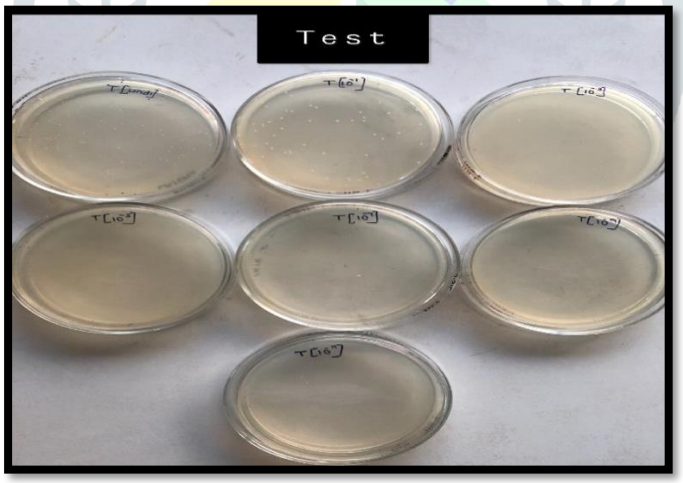
Figure7: (a) C.albicans (b) Test(ACV+ C.albicans) on Egg yolk agar medium (c) Test(ACV+ C.albicans on Milk agar plate.

Table 3:

Eggyolkagar	Zone diameter
Control	14mm
Test 30 mins	6mm
60 mins	4mm
90 mins	0mm
180 mins	0mm

5. Test for inhibition of biofilm formation i.e. adherence to acrylic resin surface:

The mean value of cfu/mL in the control group was 3×10^5, whereasthevalues for standard and test were $8.1 \times 10^2$and 5.9×10^4 respectively. Chlorhexidine presented higher reduction followed by apple cider vinegar.The mean value of cfu/ml indicates that both test and standard solution could significantly prevent the formation of mature biofilms.



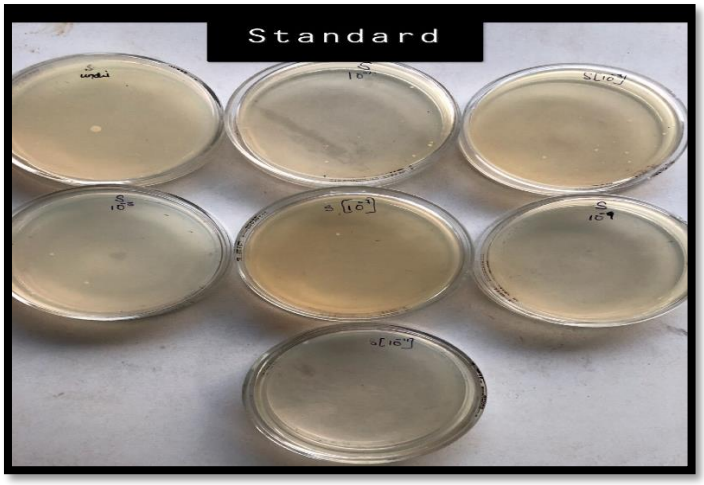


Figure 8: Screening of inhibition of biofilm formation.

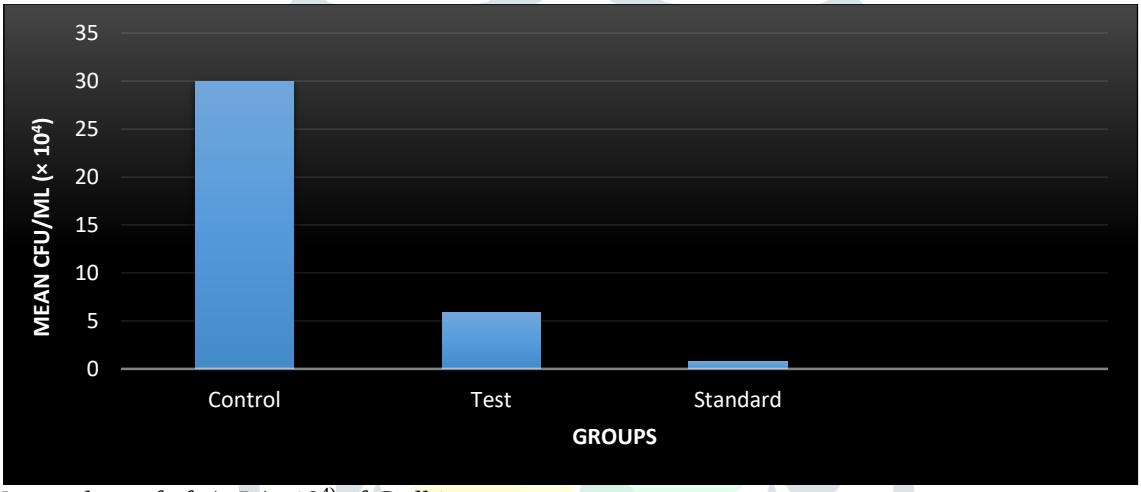


Figure 9:Mean values of cfu/mL(× $10^{4)}$ of C.albicans per group.

6. Determination of phagocytic capacity of monocytes by Flow Cytometry.(expected results):

ACV has immunomodulating activity; therefore an increase in monocyte phagocytic capacity is expected in the presence of ACV compared to the resting unstimulated monocytes. An increase in the percentage of side scatter would suggest that ACV can increase phagocytic potential in monocytes which is significant as microbial phagocytosis is a key effector function of innate immunity.

V. Discussion:

C.albicans is an opportunistic pathogen and known to be involved in denture stomatitis .Although chlorhexidineis a potent antiseptic, which is widely used for chemical plaque control in the oral cavity and as an irrigating solution, it also presents some side effects such as toxicity, burning sensation, teeth staining, disagreeable smell, and taste. In spite of the use of chlorhexidine gluconate and sodium hypochlorite as the two most popular root canal irrigates, a majority of root canal failures are caused by C.albicans. All of these facts justify the need for new therapies that could minimize the undesirable consequences of the conventional agents.In this study, apple cider vinegar at 2500 μg/ml (corresponding to 0.25% maleic acid)showed anti Candida activity whereas Chlorhexidine, showed a MIC of 7.8 µg/ml.The MIC and MFC values of ACV were found to be same indicating that ACV has strong fungicidal potential against C.albicans.One of the most important virulence factors of Candida albicans is its ability to form biofilms, which has an important clinical consequenceas it confers resistance to antifungal therapy and capacity for yeast cells within the biofilms to withstand host immune defenses. The adherence of *Candida* spp. to the surface of the acrylic resin is usually the first step in the formation of biofilms on dentures. Apple cider

vinegar and chlorhexidine were able to reduce fungal adherence to the acrylic resin as compared to control, thus indicating that it can be used to control formation of biofilms on dentures.Formation of germ tube by Candida albicans has an important consequence on its survival in different conditions and its deep penetration into the epithelia and endothelia of human tissues.The pathogenicity of Candida albicans is also attributed to certain virulence factors, such as the ability to evade host defences, and production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin. Formation of germ tubes was inhibited by ACV.ACV also inhibited the activity of the virulent enzymes, proteinase and phospholipase of C. albicans, which contribute to the pathogenicity of Candida albicans. The immune-modulatory activity of ACV is due to its ability to downregulate cytokine expression and upregulate mononuclear phagocytic function.

VI. Conclusion

Apple cider vinegar has strong fungicidal activity. ACV was found to inhibit the enzymes such as proteinases and phospholipases that contribute to the virulence of C.albicans. The formation of germ tubes, which is thought to be important for the virulence of C.albicans was inhibited by ACV. ACV also prevented the formation of biofilms, which is the major cause for drug resistance. Therefore it can be concluded that apple cider vinegar could be considered as a safer alternative for treating denture stomatitis and for root canal irrigation. There is a need for further studies to be conducted in order to evaluate its anti-inflammatory property which would expand its use in wound dressing and healing.

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