Proceeding of the Third International & the Fifth Scientific Conference of College of Science - Tikrit University
Int.3, Loc.5, Article ID:2022-P2-02

Purposeful scientific research is a basic pillar for the advancement of Iraq

In vitro Comparison of the Effect of Zinc Oxide Nanoparticles and *Hibiscus sabdariffa* Extract on *Streptococcus mutans* Isolated from Human Dental Caries

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ABSTRACT

Streptococcus mutans has been linked to dental cavities, a global public health issue. This is considered the possible cause of infection. Nanotechnology has recently been used to create unique material characteristics. The study compares the effects of aqueous and alcoholic hibiscus extracts, as well as zinc oxide nanoparticles, on S. mutans obtained from the dental cavity. Hibiscus and zinc are widely utilized in conventional therapies, along in addition to in the production of dental care products to overcome the bacterial antibiotic resistance issue. Findings have shown that hibiscus and small amounts of nano zinc oxide had a good efficacy towards *S. mutans*. To correlate zinc to the rest of the nanoparticles, it possesses a strong attachment readiness for hominid tissue which could not get damaged via zinc. In this research, the anti-bacterial effect of different amounts of ZnO NPs, and hibiscus extract against 31 *S. mutans* dental isolates. The effective sensitivity test that has been conducted along in addition to the rise of mount ZnO NPs, and the hibiscus extract showed great efficacy. ZnO NPs showed an MIC of 0.312 mg/ml while the alcoholic and aqueous extract of hibiscus showed an MIC of 12.5 25mg/ml, respectively against *S. mutans.*

Keywords: ZnO NPs, *Hibiscus sabdariffa*, *Streptococcus mutans*, MSB agar, Inhibition Zone, MIC, tooth cavities.

1. Introduction

Any action of nanoparticles on individual well-being differs from those of macromolecules [1] although the action of raised nanoparticles could possess a positive and/or negative activity [2,3]. Nanotechnology has currently drawn the world's interest due to its attributes in contrast to the large molecules from which it is created. ZnO NPs are broadly utilized for industries that include a variety of products [4], one of which is in producing antimicrobials [5] due to the effect of these nanoparticles on several species of bacteria such as *E. coli, and S. aureus* [6]. The bioactivity of inorganic oxide metal molecules such as magnesium, titanium, silicon, and zinc oxides suggests that they have the potential to be widely utilized in nanomedicine [7]. This is due to the nanoparticles' efficacy against resistant species, stability at high temperatures, and unhazardous [8]. This is highly seen in ZnO NPs activity which has been observed during the process of getting various nanoscale sizes of zinc oxide particles [9]. *Hibiscus sabdariffa* is a local bush of the Malvaceae family of the ancient torrid zone and is utilized to manufacture phloem fiber and extracts. It is an enduring shrub via pant ranging around 2m in height. The leaves comprise a wide variety of beneficial substances such as iron, niacin, and thiamine [10]. The petal produces a yellow pigment, which comprises daphniphylline. It comprises flavonoids such as hibiscitrin and hibiscetin and alkaloids such as β-sitosterol, anthocyanin, stearic acid, and wax. It is often used in the preparation of foods and drinks. Its properties vary from being a good toner to sedative a numerous portions of it have been advised for various conditions such as problems of high blood pressure and temperature, and some digestive problems in Indian alternative

medicine history. Hibiscus, notably Roselle, has been utilized in herbal remedies for heart, and malignant illnesses, in addition to being utilized as antimicrobial medication [11] Dental caries remains one of the highly well-known and prevalent oral illnesses among all ages [12,13]. *S. mutans*, a normally found bacteria in the mouth, is the major reason for teeth cavities and the highest-known bacteria having the potential to create biofilms [14, 15]. The goal of the current study is to explore some activities of nanoparticles of zinc oxide and hibiscus decoction on dental caries causing *S. mutans*.

2. Materials and methods

2.1. Specimen gathering and isolation of bacteria:

The specimens have been gathered from the dental cavities of the 135 patients in Tikrit city clinics aged from 8 to 65 of both genders. The specimens have been gathered via cotton swabs to obtain *Staphylococcus and Streptococcus*, which have been cultured in Mannitol salt agar and Mitis salivarius bacitracin agar. The media have been incubated in standard condiotions. After that, 31 isolates of *S.mutans* from all the bacterial cultures in the media have been differentiated using the typical methods of culture differentiation including biochemical testings, staining, and outer emergence differentiation of the colonies *Staphylococcus* and *Streptococcus* [16].

2.2. Preparation of Mitis Salivarius Bacitracin Agar (MSB) medium

The selective medium MSB medium for *S. mutans* in Figure 1 has been set as stated via Hi-media 's instructions using 20(w/v) % total percentage of sucrose and 200IU / L of the antibiotic Bacitracin and the medium has been solidified using 0.0001 Potassium tellurite solution [17, 18, 19]. A solution of 90 gm/L of the medium has been set via it into D.W and mixing the two thoroughly. Then 150 gm/L of Sucrose has been added and mixed. After the media turned to transparent blue, they have been autoclaved at standard conditions set via the manufacturer. Filtered potassium tellurite 0.0001 at 45 °C (Fishr Scientific, Wein, Austria) [16]. Bacitracin (1 ml) has been set via solvating 0.364 of the antibiotic material in 100 ml of Distilled Water then the mixture has been thoroughly mixed then filtered to be stored at 200IU / L conc. The solution has been added to the media prepared at a percentage of 1:1000[17].

Fig. 1: MSB Agar plate

2.3. Muller Hinton Agar (MHA) Setting

This medium (Hi-media) has been set as stated via the manufacturer [20] via dissolving 38 gm of the medium material into 1 L of D.W thoroughly. Then the medium has been autoclaved at standard conditions set via the manufacturer and poured into plates till it cooled to be stored for use.

2.4. Isolation of *Streptococcus mutans*

Streptococcus mutans has been obtained using the procedure outlined via [21] which is gathering specimens in addition to cotton swabs from the infected areas of the oral cavity. The isolates have been cultured on MSB and incubated at standard conditions. After that, they have been cultured on blood agar to get the *S.* in a standard condition of an anaerobic jar for a day. Then the colonies have been cultured again on an MSB medium and they have been then preserved in an anaerobic container for 2 days in standard conditions.

2.5. *Streptococcus mutans* identification

1- A morphological feature:

S. mutans has been differentiated via the colony shape on MSB agar, its outer emergence under microscopic testing, features stated via Edwardson [21,22], and the type of hemolysis on the blood agar plate [23].

2-Gram staining

A small number of colonies from MSB Agar have been dyed in addition to Gram stain in uncontaminated circumstances (SYR BIO, England) as followed via microbiologists [24].

3- Biochemical Tests

A-Catalase test

Each colony of *S. mutans* has been mixed in addition to a drip of 3% H_2O_2 (Panreac, Barcelona, Spain) on an uncontaminated part (Citotest, Nantong, China). What indicated the positive result has been the release of air [25,26].

B- Test for carbohydrate fermentation (mannitol fermentation test) This has been carried out via observing the change of color of the Cystine-trypticase- agar CTA-mannitol medium from red to yellow, which is considered to be a positive indicator for the presence of *S. mutans* that possesses the ability of mannitol fermentation [27].

2.6. Diagnosis of *Streptococcus mutans* via VITEK-2

Streptococcus mutans bacteria have been differentiated via VITEK-2 (BIO meriex) following the culturing of the specimens on MSB, as stated via the manufacturer's directions [28].

2.7. Zinc oxide nanoparticle characterization

ZnO NPs in addition to diameters of 20-30nm and purity of 99.98% have been supplied from U.S. Research Nanomaterial, Inc (USA), and the standard solutions have been prepared at an amount of 10 mg/ml and diluted according to c1v1 = c2v2[21]. The nanocomposite testing has been carried out in addition to the use of a scanning electron microscope (SEM) which can be seen in figure 2.

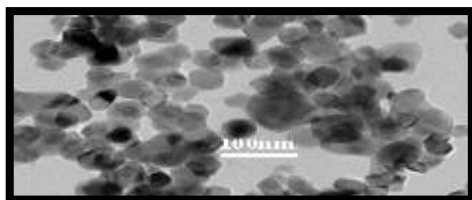

Fig. 2: Scanning Electron Microscope (SEM) showing Zinc oxide nanoparticles.

For verifying the efficacy of the ZnO NPs mixture, an exam via the UV-V machine is performed, which figure 3 shows it, and the greatest absorbance has been 377 nm, proving how such a substance is nanoscale, and the absorption should rise to the greatest value has been 360 - 500 nm, as noticed when testing such particles via UV/visible spectroscopy.

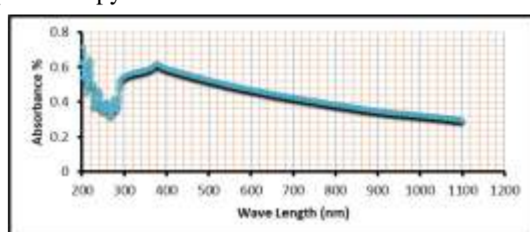

Fig. : 3 ZnO NPs shown via UV/visible spectroscopy

2.8. Aqueous shrub extracts preparation:

These extracts have been obtained via mixing 40 gm of the shrub in 160 ml of D.W., which will be in a 4: 1 w / v ratio. The shrub has been inserted in the mixer in addition toin an extremely cold condition, then the combination has been mixed in addition toin at least 60 minutes in addition to a magnetic stirrer to examine and destroy the shrub cell wall. The combination of the shrub has been then refrigerated for 24 hours to dunk thoroughly before being strained. The combination has been filtered via many layers of gauze and filter paper to separate the remains. The crude aqueous extract then has been set, and then it has been dehydrated via the pressure of a lypholizer device, and then the specimens have been dehydrated under zero humidity conditions to be set aside for future use [29].

2.9. Alcoholic shrub extracts preparation:

In the setting of alcohol extracts, the researcher's approach [31] has been used, which involved smashing (50) grams of the shrub in 200 ml of ethyl alcohol at an amount of 95% in extremely cold conditions, followed via vigorous shaking in addition to the magnetic stirrer apparatus. After 24 hours in the cooler, the combination has been filtered via many layers of fabric to separate the alcohol, then the combination has been put in the Rotary Evaporator, which is based on the principle of evaporation at low pressure and heat degree not surpassing 40 ° C, then the residue has been evaporated. Layers of alcohol have been evaporated via subjecting them to cold conditions in scarce pressure via a lyophilizer device. The specimen has been then dried via putting it in glass bottles in addition to tight sources in maximized dry circumstances. The aqueous shrub extracts have been kept via cooling in extremely cold conditions till utilization [30].

2.10. Aqueous extracts sterilizing:

The sterilization of the aqueous extracts has been conducted according to what has been stated in [31] which included setting a storage solution of 200 mg/ml via mixing 1 g of the dried aqueous extract in addition to 5 ml of D.W. This mixture has been filtered through a 0.22 µm membrane. The mixture has been utilized in the setting of several amounts 10, 25,50,75, and 100 utilized in the study.

2.11. Alcoholic extracts sterilizing:

The sterilization of the alcoholic extracts has been conducted according to what has been stated in [32] which included setting a storage solution of 200 mg/ml via dissolving 1 g of alcoholic extract in 5 ml of Dimethyl Sulphoxide. Combinations have been pasteurized at 62°C For 10 minutes. This solution has been used in the setting of the several amounts used in the research.

2.12. Fourier transforms infrared spectroscopy of shrub extract (FTIR)

This approach has been utilized at Tikrit University's/ College of Science/Dept. of Chemistry. The extracts' reflective infrared spectroscopy has been calibrated at 400-4000 using potassium bromide disks to broaden the range to 25 Micron (400 Cm-1). 10 cc of dimethyl sulfoxide has been combined in addition to 0.01 g of extract. The mixture has been subjected to ultrasonography to increase the process of mixing and then turned into solid particles via hydraulic press high pressure.

2.13. Determination of *Streptococcus mutans* sensitivity to different amounts of ZnO NPs mixtures and de-ionized water *(In vitro)*

The efficacy of ZnO NPs has been examined against the tooth cavities *Streptococcus mutans* obtained as stated in NCCLS [33], which comprised the upcoming steps: preparing a bacterial suspension of *S. mutans* isolates, which is supposed to be examined in addition to different amounts of ZnO NPs. Streptococcus mutans microbial suspension has been created via combining 3 colonies and 5 ml of N.S and then comparing the microbial suspension to a typical McFarland solution (0.5) to maintain a constant cell number of 1.5 x 108 cells/ml. Following that, 100 1 of the microbial b suspension has been collected and placed over the surface of Muller Hinton agar in addition to specific dimensions to disseminate the suspension adequately. Several 5 mm wells have been formed in MHA using a cork puncher (HI-Media, India). The wells have been filled in addition to a number of amounts of ZnO NPs nanoparticles. A negative control has been established from de-ionized water in these amounts: 10 mg/ml, 5 mg/ml, 3 mg/ml, 1 mg/ml, 0.5 mg/ml, and 0.1 mg/ml.

2.14. ZnONP Minimum Inhibitory Amount (MIC) Measurement

via broth watering down , it is significant to decide the minimum inhibitory amount of zinc oxide in a nanoscale approach. The microbial suspension has been set via culturing a few colonies in broth for a quarter of an hour. The microbial suspension should be thoroughly mixed. 0.1 ml of this suspension has been applied to several tubes comprising 4.8 ml of the nutrient broth and 0.1 ml of zinc oxide solution that has been set and added as in the amounts 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 mg/ml in addition to a positive control tube that comprises broth and inoculum of bacteria to discover the capacity of the bacteria to grow on the nutrient broth. The negative control tube comprised just the nutrient broth and ZnONPs in addition toout bacteria. The inoculated tubes have been preserved till the second day in standard conditions. After that, the growth in the tubes has been contrasted against control tubes, where the first tube contained the minimum inhibitory amount in addition to no visible turbidity [34].

2.15. *Streptococcus mutans* sensitivity calculations of different amount of Hibiscus shrub extracts mixtures *(In vitro):*

The distribution method has been utilized to produce three subsequent amounts according to what has been stated in [35]. 50 micro-liters of the shrub extract have been inserted into the wells in addition to amounts 10, 100,75,50,25 mg/ml. Distilled water and DMSO solution have been inserted into the standard well of aqueous extract and alcoholic extract, respectively. The tubes have been incubated for a day at a typical temperature of 37 ° C, and the efficacy of each watering down of the shrub extracts has been determined via measuring the length of the inhibition zone in mm via a standard ruler.

2.16. Evaluating MIC of Hibiscus Extracts

The MIC is a significant step in deciding the MIC against microbes used in this study. It is likewise regarding the MIC of the shrub extract which has been measured using the broth watering down way. First, the microbial suspension has been set via obtaining a few colonies and inserting them into the broth. After a quarter of an hour, the inserted colonies have been mixed and 0.1 ml of the microbial suspension has been inserted into high temperature testing tubes containing 9.8 ml of the nutrient broth then 0.1 ml of the shrub extract that has been set in various amounts has been inserted (0.78,1.57, 3.125, 6.25, 12.5,25, 50, 100) Mg/L. One of the tubes in the watering down sequence has been a positive control tube which comprised just broth and the inoculation while the negative control tube contained just broth. The inoculated tubes have been set for incubation at a standard condition for a day. Growth has been noticed in all the tubes except for the tubes that contained the lowest amount where the has been no visible microbial turbidity [36].

3. Findings

3.1. Morphological characteristic: *Streptococcus mutans* have been tested depending on the outer emergence using magnification (Olympos, Japan). The colonies have been blue, 1-2 mm in diameter, and had a rounded shape in addition to some raised areas, since the polysaccharides have been aggregated in the middle of the colonies, as seen in figure 4.

Fig. 4: Growth form of *S. mutans* colonies on MSB Agar plate

3.2. Testing under magnification: Under the oily lens, *Streptococcus mutans* appeared to be violet, high from the side, rounded, in addition to chains that are not long, as seen in figure 5.

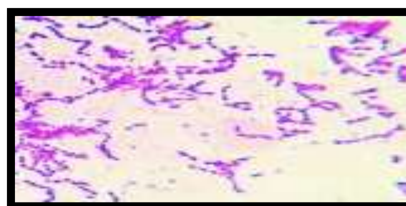

Fig. 5: *S. mutans* gram-positive stain

3.3. Biochemical Tests:

A-Catalase test

Sine *S. mutans* do not possess the capacity to form catalase, findings have been negative in addition to no globules of air forming from the reaction in addition to H_2O_2 3% as seen in figure 6.

Fig. 6: Catalase test for *S. mutans*

B- Carbohydrate fermentation test (mannitol fermentation test): Since the hue of the media has shifted from red to yellow. The testing has been positive for *streptococcus mutans* as seen in figure 7.

Fig. 7: *S. mutans* – mannitol fermentation testing. A: Negative control tube (agar in addition to mannitol free of bacteria). B: Study tube (agar in addition to mannitol inoculated via *Streptococcus mutans*). C: Positive control tube (bacteria in addition to agar free of mannitol).

3.4. *Streptococcus mutans* detection via VITEK-2: The result using VITEK2 has been almost definite for presence *Streptococcus mutans*, since it has been 96% as seen in figure 8.

Organism Quantity :	Selected Organism : streptococcus mutans		
Comments:			
Identification information	Card: GP	Lot Number : 2421207246	Expires : Mar 17,2021 13:00 CDT
Organism origin	VITEK 2		
Selected organism	96% Probability		Streptococcus mutans

Fig. 8: VITEK-2 test findings

3.5. FTIR findings of Hibiscus extracts

The IR findings of the hydrophobic extract showed the emergence of a strong bundle which has been seen in (3477) cm^{-1} because of OH group. The hydration in addition to the emergence of a suction bundle has been at range (2928) cm^{-1} because of the aliphatic CH group, and the emergence of 2 suction bundles at (1797 and 1741) cm^{-1} due to two carbonyls (C=O) which equal the emergence of 2 suction bundles at the range of (1618, 1431) cm^{-1} belongs to the (C=C) aromatics, in addition to the emergence of another bundle at (1226) cm^{-1} due to (C-O), and the bundles' emergence at (1022) cm^{-1} relates to the assigned mat (C-N) [37], as shown in figure 9.

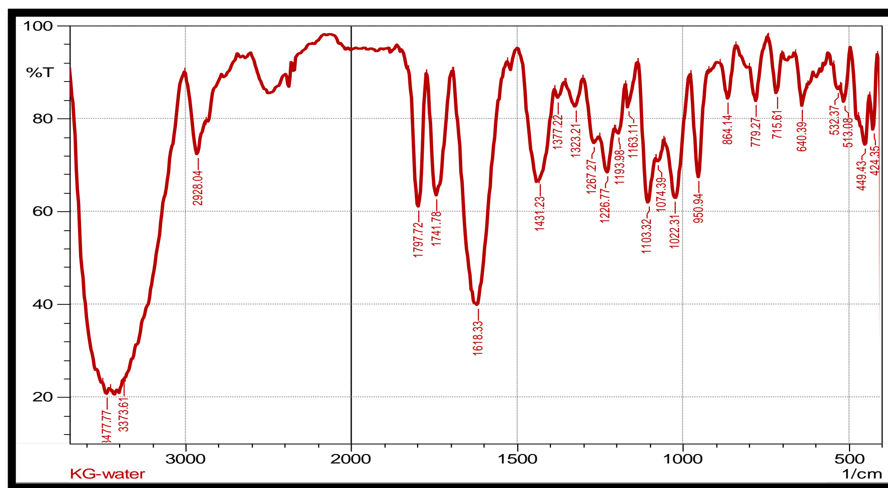

Fig. 9: FTIR findings of Hibiscus aqueous extract

The findings of the IR spectrum of alcoholic extract, a strong bundle which has been seen at (3429) cm^{-1} due to the (OH) group of the alcohol and the emergence of suction bundles at (2924) cm^{-1} due to the aliphatic group (CH). There has been an emergence of a bundle at (1793 and 1745) cm^{-1}, (C=O) in addition to the emergence of two suction bundles at range (1618, 1429) cm^{-1} due to the (C=C) aromatics, in addition, there has been an emergence of a bundle at (1224) cm^{-1} due to (C-O), and an emergence of a bundle at (1024) cm^{-1} belongs to a determined mat. (C-N) [38], as seen in figure 10.

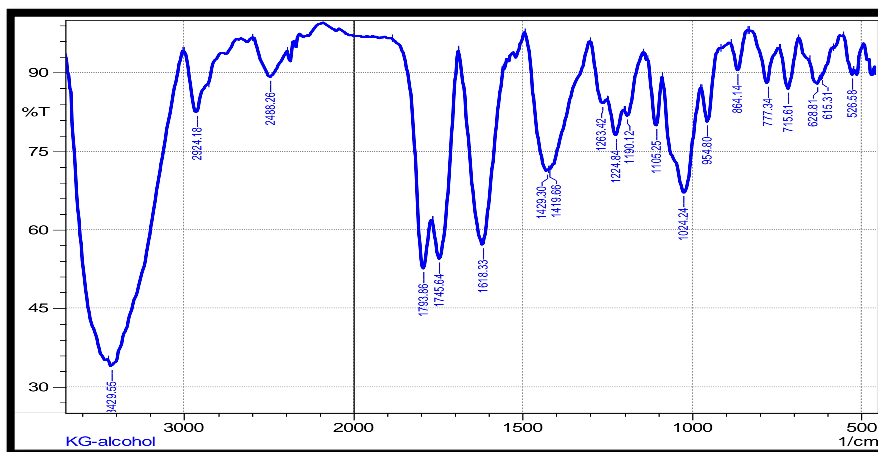

Fig. 10: FTIR findings of Hibiscus alcoholic extract

3.6. Estimation of *S. mutans* sensitivity to different amounts of mixtures of hibiscus shrub extracts *(In vitro):*

The amount of 25 mg/ml of hibiscus aqueous extracts showed positive findings against *S. mutans* which has the least inhibition diameter, however, the amount of 100 mg/ml showed the best efficacy shown in Figure 11.

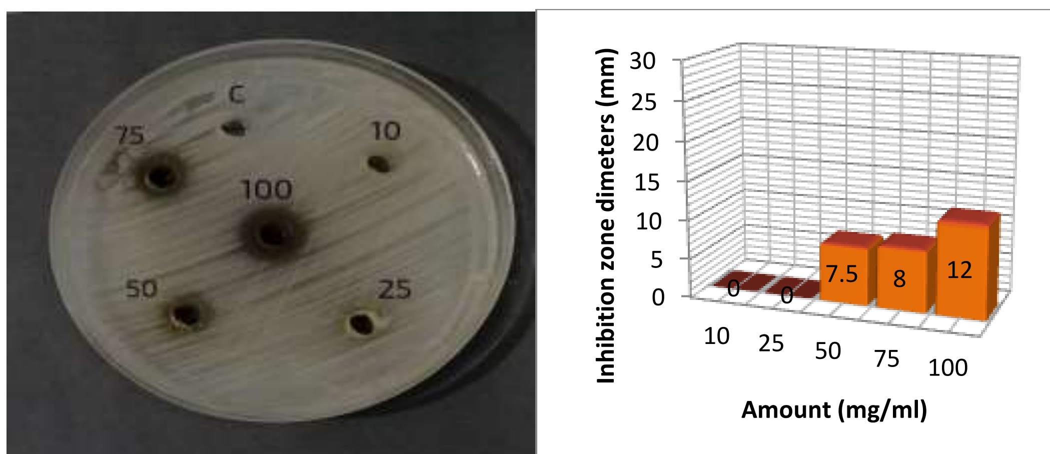

Fig. 11: ***Streptococcus mutans*** **susceptibility to various amounts of aqueous hibiscus extracts.**

At a amount of 25 mg/ml of hibiscus alcoholic extracts against *S. mutans*, the findings showed the least efficacy, however, at the amount of 100 mg/ml the findings showed the best efficacy as seen in Figure 12.

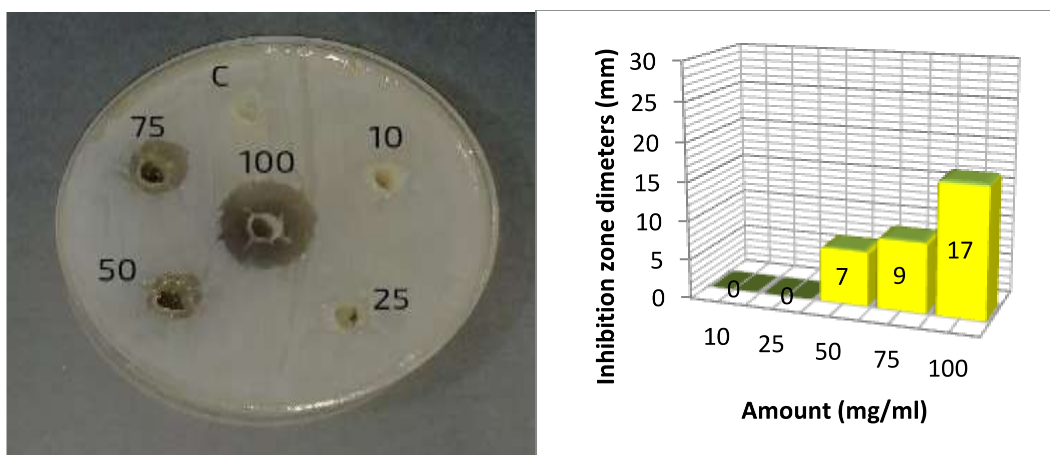

Fig. 12 Susceptibility test of *Streptococcus mutans* to various amounts of alcoholic hibiscus extracts

has been clear. While tube No 3 c a comprising an amount of 25 mg/ml of alcoholic hibiscus extracts showed more clarity, as seen in table 1.

3.7. MIC evaluation of hibiscus extract

After a day of incubation, tube No 4 in addition to a amount of 12.5 mg/ml for the Aqueous extract of hibiscus showed the lowest growth amount since it

Table 1: MIC of Hibiscus extract on *Streptococcus mutans*

Tube No.	+ C	C–	1	2	3	4	5	6	7	8	MIC
hibiscus extract amount	0 mg/ml	0.39 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml	1.57 mg/ml	0.78 mg/ml	
Aqueous extract	+	–	–	–	–	+-	+	+	+	+	25mg/ml
Alcoholic extract	+	–	–	–	–	–	+	+	+	+	12.5mg/ml
= absence of growth, + = presence of growth, - C = Negative Control, + C = Positive Control –											

3.8. Evaluation of *S. mutans* sensitivity of different amounts of ZnO NPs solution and de-ionized water (*In vitro*)

Results of the *S. mutans* sensitivity testing against ZnO NPs have been observed about 0.5 mg/ml which has been the least efficacy, while at 10 mg/ml the best efficacy which is shown in figure 13.

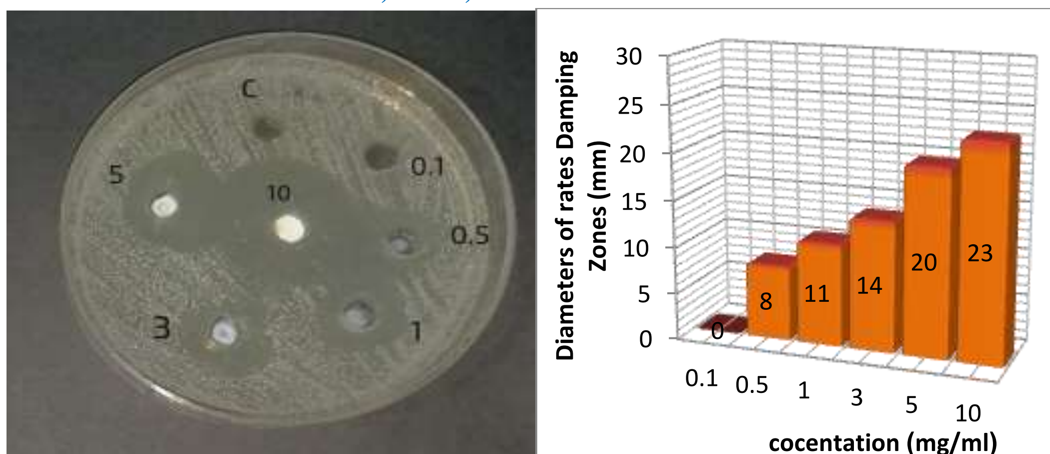

Fig. 13 ***S. mutans*** **susceptibility against various amounts of ZnO NPs**

3.9. Measuring the Minimum Inhibitory Amount (MIC) of ZnONPs

MIC has been decided on the next day of incubation, in addition to the lowest amount of growth inhibition has been seen in Tube No. 6 in addition to 0.312 mg/ml amount of ZnO NPs as seen in table 2.

Table 2: MIC of ZnONPs on ***S.mutans***

Tube No.	+ C	C–	1	2	3	4	5	6	7	MIC
Amount ZnO NPs	0 mg/ml	0.078 mg/ml	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml	0.312 mg/ml	0.156 mg/ml	
Growth	+	–	–	–	–	–	–	–	+	0.312 mg/ml
= absence of growth, + = presence of growth, - C = Negative Control, + C = Positive Control –										

4. Discussion

The quantitative assessment of the antimicrobial nanoparticle has been detected via distribution in plate wells. It has been discovered that the inhibitory region is mostly determined via the amount, which is consistent in addition to [39]. This demonstrates that raising the amount of ZnO NPs enhanced bacterial inhibition, and results have been shown to be similar to those stated in [40], who discovered that zinc particles Nanoparticles possess the highest effectivity towards the action of Gram-positive and Gram-negative bacteria, and ZnO NPs possess outstanding antimicrobial effect. Elevation of the inhibitory region in addition to the increasing amount might have been due to ZnO NPs, which have a larger surface-to-volume ratio, resulting in improved ion swap. The least amount of ZnO NPs tested has been 0.1 mg/ml, which had no effect on S. mutans bacteria isolated from dental cavities. Numerous processes of anti-microbial influence via ZnONPs and the action of such substances via producing damaging oxygen substances to living cells, H_2O_2 generation, and if the amount of zinc oxide nanoparticles is increased, its findings in H_2O_2 elevation would in return increase the antimicrobial efficacy. This consequence happens as a result of cell membrane disruption, which produces a fault in the cell's components too, resulting in the cell's irreversible death [41]. Certain research has found that the anti-bacterial effectiveness of ZnO NPs is due to their tiny size, which is 250 times smaller than a bacterial cell and allows these particles to bind to the microorganisms' walls, resulting in the disintegration and death of live cells. An additional reason for ZnO NPs' antimicrobial action is the production of Zn+ 2 ions, which have the capacity to decompose cell membranes and react with intracellular contents [21]. The electron microscope pictures revealed that ZnO NPs had an influence on the bacterial cell wall, altering the permeability of the cell membranes, and modifying the cell shape. This has been presumed to be caused by the interaction of ZnO NPs in addition to the bacterial cell membrane, leading to a defect in the membrane function. This alters the permeability of the membrane, resulting in intracellular constituent release and then apoptosis [41]. This contact has been most probably caused via electrostatic effects caused via the opposing polarities of the nanoparticles and the cell membrane. According to one research [21], when ZnO NPs interacted in addition to the bacteria, their toxic activity causes the bacterial bilayer lipid layer to tear, resulting in cytoplasmic spillage, and another probable way to suppress the bacteria. Weak DNA damage has been seen in the treated bacteria [42]. It is thought that micro-nanostructures have a negative charge whereas metal oxides have a positive charge, which causes the electromagnetic affinity between the germ and the exterior of the zinc nanoparticles to be modulated. When this attraction happens, the microbe is oxidized and killed rapidly, and it is generally assumed that the formation of ions via nanomaterials that react in addition to the reliant SH groups is responsible. Regarding polypeptides on the bacterial exterior of the cell [43]. The antimicrobial effect of *H. sabdariffa* cyclic extracts

can be linked to the phytochemical constituents found in them [44]. These bioactive chemicals are known to have antibacterial activity in a distinct manner. Flavonoids are hydroxylated phenolic chemicals that shrubs produce as a consequence of microbial invasion, and they have been shown in vitro to be efficient antibacterial agents against a wide range of pathogens. Their action is most likely related to their capacity to interact in addition to extracellular and soluble proteins, as well as bacterial cell walls [45]. These findings are consistent to what was stated in [46], who discovered that shrub polyphenols have been shown to possess antibacterial properties. Polyphenolic chemicals and/or volatile oils have been shown to inhibit several organisms [47]. The antimicrobial effect of gossypetin derived from H. sabdariffa has been studied, and the action may be attributed to the flavonoid gossyypetin's polyphenolic characteristic [48]. This chemical is highly effective against germs that lead to food poisoning, infections, and dental problems including dental cavities [49].

5. Conclusion

The findings show that nanoparticles of zinc oxide and hibiscus extract possess strong antimicrobial properties, and the efficiency of these particles rises as the amount of the solution containing the nanoparticles rises.

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