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Preparation of Mouthwash using Vitis Vinifera-Carbon Nanoparticles and its Biological Evaluation - An Invitro Study

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Nanoparticles are materials with dimensions of 1 to 100 nm. They have various properties due to their size, which can be used for the development of humankind. Carbon nanoparticle is one of the important nanoparticles because of their magnificent properties and they are less toxic. So, they can be used to prepare a mouthwash. They are synthesized by green synthesis because it involves using renewable, less toxic and cheap raw materials which are safe for humans and the environment. The plant chosen for synthesizing nanoparticles is Vitis vinifera. Grape (Vitis vinifera) has been consumed as food for many years. It is known to have many medicinal properties. The objective of the study is checking the effectiveness of mouthwash prepared using Vitis vinifera carbon nanoparticles. The effectiveness of the prepared mouthwash is tested on four dental pathogens (S. aureus, C. albicans, Lactobacillus sp., and S. mutans) with different concentrations of nanoparticles and compared with commercial mouthwash. The assays used for assessment are agar well diffusion assay and time kill curve assay. Anti-inflammatory was tested using BSA assay, Membrane stabilization assay and egg albumin assay. The results vary with different pathogens. The maximum zone of inhibition by carbon nanoparticles of 11mm is obtained against S. mutans at 100µg/ml concentration followed by 10mm against the same pathogen at 50µg/ml. The antimicrobial properties of nanoparticles increased with their concentrations. Commercial mouthwash performed better than the nanoparticle against S. aureus with a zone of inhibition of 21mm. Anti-inflammatory assays show a concentration-dependent effect and the standard performed better than carbon nanoparticles at all concentrations. From the study, we could conclude that we can successfully synthesize an effective mouthwash from Vitis vinifera carbon nanoparticles. It has potent antimicrobial and anti-inflammatory properties. Further studies can be done to improve the effectiveness of mouthwash.

Keywords: Antimicrobial, carbon nanoparticles, green synthesis, Mouthwash, nanoparticles, Vitis vinifera.

1. Introduction

Nanoparticles are defined as insoluble and bio-persistent materials with dimensions ranging from 1 to 100 nanometers. Nanoparticles can be used as colloidal drug carrier systems. Nanoparticles have benefits like the sustainable release of drugs, more species-specific, and a high surface volume ratio ((Rashki et al., 2021)). Nanoparticles exist in nature and as artificially synthesized by humans. They have unique characteristics because of their microscopic size. These particles have many practical applications in various fields including medicine. Nanoparticles are incorporated into composite material (Oake et al., 2019). Synthesis of nanoparticles using physical and chemical processes is costly, with excess energy demand and usage of toxic substances. Approaches of biological synthesis of nanoparticles are classified into two approaches: the top-down approach and the bottom-up approach. The top-down approach is associated with reducing the size of large particles to nanoparticles. The bottom-up approach refers to building up the size of particles (Qiao et al., 2022). Green synthesis of nanoparticles is preferred because it is cheap, uses nontoxic raw materials, and renewable sources, and is eco-friendly. Green synthesis is emerging in nanotechnology. The products used for synthesizing nanoparticles are attracting researchers because of their feasibility, eco-friendliness, and their infinite availability. The nanoparticles synthesized by green synthesis have extraordinary structural properties, size distribution, etc (Manikandan & Lee, 2022).

Carbon is a mineral that is abundant on Earth. Many molecules that are essential for life are made up of carbon. Carbon nanoparticles were first discovered in the 1980s. Carbon nanoparticles have excellent stability, heat conductivity, electrical conductivity, less toxicity, eco-friendliness, and biocompatibility because they are formed from pure carbon. Carbon nanoparticles are used in many industries like agriculture, cosmetics, medicine, and electronics. Carbon nanoparticles are one of the most researched nanoparticles. These carbon nanoparticles have high stability and less toxicity. There are many kinds of carbon nanoparticles like graphene, fullerenes, nanotubes, etc. (Holmannova et al., 2022). Carbon nanoparticles are used as antibacterials because of the global spread of resistance against antibacterials by bacteria (Chandra et al., 2011).

In India, many people suffer from tooth decay and periodontal disease. Brushing teeth is an important way to prevent these kinds of oral diseases. Brushing also reduces the risk of getting plaque. However, brushing is difficult and not possible for paralyzed, disabled, or traumatized patients. Mouthwash plays an important role for these patients to prevent them from oral diseases. Traditional mouthwashes like chlorhexidine have side effects like discoloration of enamel, mucosal irritation, and changes in the taste of food. So, we need a natural alternative. (Abadi et al., 2013). Mouthwashes are also claimed to have anti-plaque and anti-calculus activity (Huang et al., 2023). Grapes (Vitis vinifera) are the oldest kind of fruit and it has been grown and consumed by humans for a very long time. It is considered an economically important fruit because it is rich in minerals, vitamins, sugars, and other nutrients (Đorđevski et al., 2022). Grapes are rich in polyphenolic compounds which make them antimicrobial to bacteria being resistant to antibiotics (Yadav et al., 2015). So, it along with carbon nanoparticle's antibacterial properties can be used to make an effective mouthwash.

The objective of this research is to prepare an efficient mouthwash from Vitis vinifera carbon

nanoparticles and to test its effectiveness in an in vitro study. Anti-inflammatory and antimicrobial property is studied to see the effectiveness of mouthwash. The antibacterial property is studied by the agar well diffusion method and time-kill curve analysis. The effectiveness of mouthwash is compared with commercial mouthwash. This helps in preparing mouthwash from natural products for human use.

2. Materials and Methods:

Preparation of carbon nanoparticles:

Vitis vinifera seeds are washed thoroughly with the help of distilled water, dried, and crushed to powder. 5g of the Vitis vinifera seeds were then combined with 100 mL of distilled water and boiled by making use of a heating mantle. The microwave oven at 700 volts is used for synthesizing carbon nanoparticles using 1 using a crucible. The residues that were settled in the crucible were then crushed well and made into a fine powder. The fine powder is then mixed with distilled water and put in the sonicator for 10 minutes. The CNPs were kept in the hot air oven to dry and used for further studies. Chitosan of 500mg was added with 1 mL of glacial acetic acid and then mixed with 49 mL of pure distilled water. A magnetic stirrer was used till the solution became a clear solution. 0.2g in 3 ml of chitosan mixed and kept in the sonicator for 15 minutes.

Preparation of mouthwash:

The mouthwash was synthesized by utilizing sucrose of 0.3g for sweetening, sodium benzoate of 0.001g as a preservative, and sodium lauryl sulfate of 0.01g as the foaming agent. In distilled water of 9.5ml, these were dissolved. Then, 500 µg/mL of chitosan-incorporated Vitis vinifera-mediated carbon nanocomposites were combined for mouthwash preparation and then the mixture was placed in an orbital shaker. Finally, for further studies, 10 mL of chitosan-incorporated Vitis vinifera-mediated carbon nanocomposites-based mouthwash was used.

Agar well diffusion method:

The antimicrobial ability of carbon nanoparticles is assessed against strains of four pathogens S. aureus, C. albicans, Lactobacillus sp, and S. mutans. Mueller Hinton agar plates are used for assessing antibacterial activity by determining zones of inhibition. Mueller Hinton agar plate is prepared and sterilized for 15 minutes at 121 degrees Celsius. Then in the sterilized plates medium was poured and left to solidify. Then the wells are dug using a sterile polystyrene tip of 9mm. Then the test pathogens are swabbed. The nanoparticles with concentrations of (25µL, 50 µL,100 µL) are poured into the three wells. Another fourth well is kept as a control. Then the plates were kept for incubation at 37 degrees Celsius for one day. After incubation measurement of zone of inhibition was done (Rajeshkumar et al., 2019; Rifaath et al., 2023).

Time kill curve assay:

The antimicrobial activity of carbon nanoparticles is assessed by time-kill curve assay. Effects of different concentrations of nanoparticles (25µg/ml, 50µg/ml, 100µg/ml) were tested on cultures of four pathogens S. aureus, S. mutans, C. albicans, and Lactobacillus sp in Mueller Hinton Broth. Commercial mouthwash was used as standard. After a pre-incubation period of

four hours without any antimicrobial agents to make sure all pathogens get to a stable early to the mid-log phase then an inoculum with 0.5 McFarland of pathogens in sterile phosphate buffered saline was created. Inoculum was collected in agar plates of Mueller Hinton at a temperature of 37 degrees Celsius up to 20 hours. Then 30µL of inoculum was mixed in Mueller Hinton Broth of 15ml that is antimicrobial free, after preheating for 37 degrees Celsius and then 90µL of the mixture is distributed uniformly over 96 well ELISA plates. Then to each well 90µL of pathogens and 10µL of chitosan-incorporated Vitis vinifera-mediated carbon nanocomposites along with untreated control was added (azhlini et al., 2021; Tharani et al., 2023).

Bovine serum albumin assay:

The bovine serum albumin denaturation assay was used to check the anti-inflammatory activity of mouthwash from green synthesized chitosan-incorporated Vitis vinifera-mediated carbon nanocomposites. Bovine serum albumin of 0.45 ml was added with 0.05 ml of variable concentrations (10, 20, 30,40, 50 µg/mL) of chitosan-incorporated Vitis vinifera-mediated carbon nanocomposites. The pH was set to 6.3. After keeping it at room temperature for 10 minutes it was kept in incubation in a water bath at 55 degrees Celsius for 30 minutes. Dimethyl sulphoxide was served as a control and diclofenac sodium was utilized as a standard. The samples are measured spectrophotometrically at 660 nm. The protein denaturation is determined using the following formula,

% inhibition= Absorbance of control- Absorbance of sample×100

Absorbance of control.

Egg albumin assay:

0.2 ml of egg albumin is combined with 2.8 ml of 1X phosphate buffer for performing egg albumin denaturation assay. Then several concentrations (10, 20, 30,40, 50 µg/mL) of carbon nanoparticles synthesized using Vitis vinifera were added to the mixture. Then pH was maintained at 6.3. After keeping it at room temperature of 10 minutes it was incubated in a water bath for 30 minutes at 55 degrees Celsius. Diclofenac sodium serves as standard and dimethyl sulphoxide works as a control. Then at 660 nm samples are measured spectrophotometrically (Sankar et al., 2024; Munusamy & Shanmugam, 2023; Subramanian et al., 2021). The following equation was used to find protein denaturation,

% inhibition= Absorbance of control- Absorbance of sample×100

Absorbance of control

Membrane stabilization assay:

The in vitro membrane stabilization assay is widely used for evaluating properties to stabilize membranes of various artificial and natural compounds. This assay works by preventing disrupting releasing intracellular contents and measuring membrane stabilization. The materials like phosphate-buffered saline, human red blood cells, tris-Hcl buffer (50 mM, pH 7.4), differing concentrations (10, 20, 30,40, 50 µg/mL) of carbon nanoparticles, centrifuge tube, UV-vis spectrophotometer are needed.

Preparation of RBC suspension

Human blood was extracted freshly in a sterile tube that contains anticoagulant in it. Then the sample was centrifuged for 10 minutes at 3000 RPM in a centrifuge at room temperature to separate RBC. Remove the supernatant and wash RBSs with the PBS three times. Resuspend the RBCs to get 10% (v/v) RBC suspension in the tris-Hcl buffer. RBC suspension of 1 ml was pipetted and put in each centrifuge tube. Then carbon nanoparticles of variable concentrations (10, 20, 30,40, 50 µg/mL) are added to each centrifuge tube and mixed gently. Then incubate tubes at 37 degrees Celsius for 30 minutes. Then the tube was centrifuged at room temperature at 2500 RPM for 5 minutes. The absorbance of the supernatant was measured at 560 nm by using a UV-Vis spectrophotometer.

3. Results:

Figure 1: Represents the synthesis of Vitis vinifera assisted carbon nanoparticles and it's incorporated chitosan nanocomposite

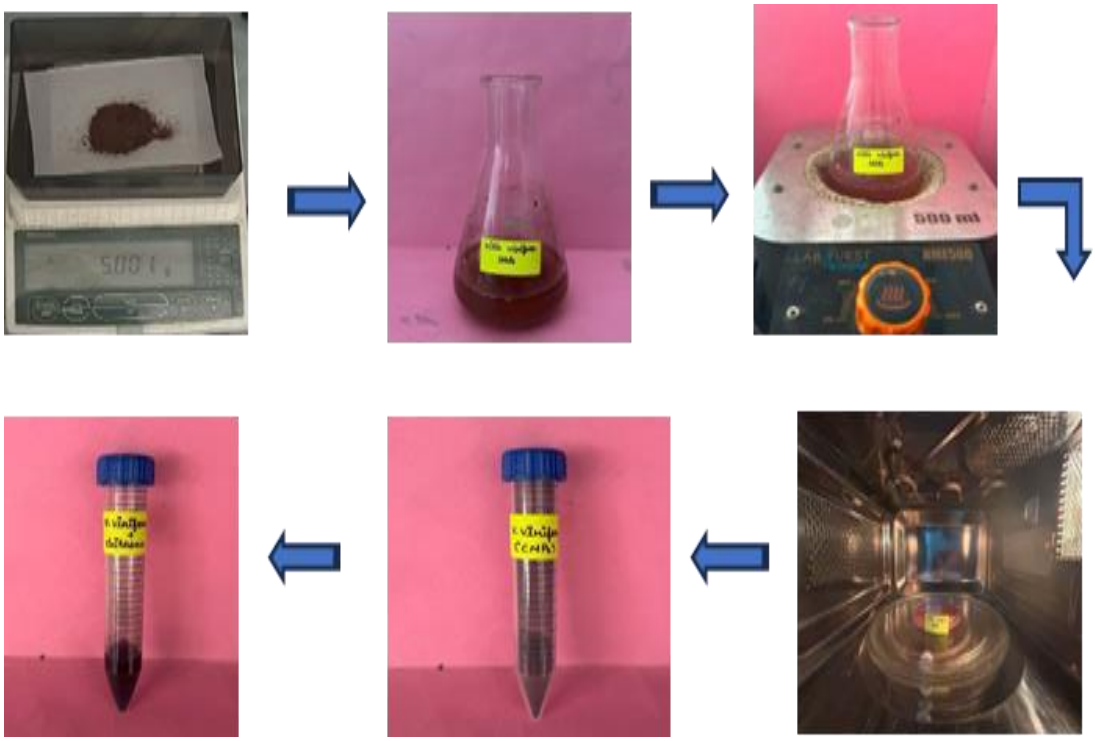


Figure 2: Antimicrobial activity of carbon nanoparticles against S. aureus, Lactobacillus sp, C.albicans, S.mutans.

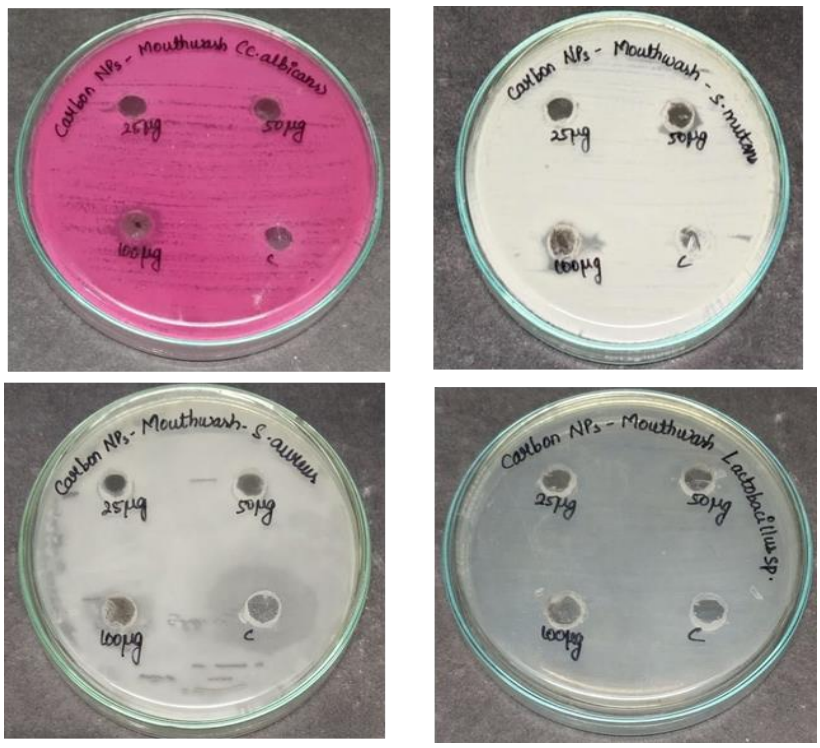
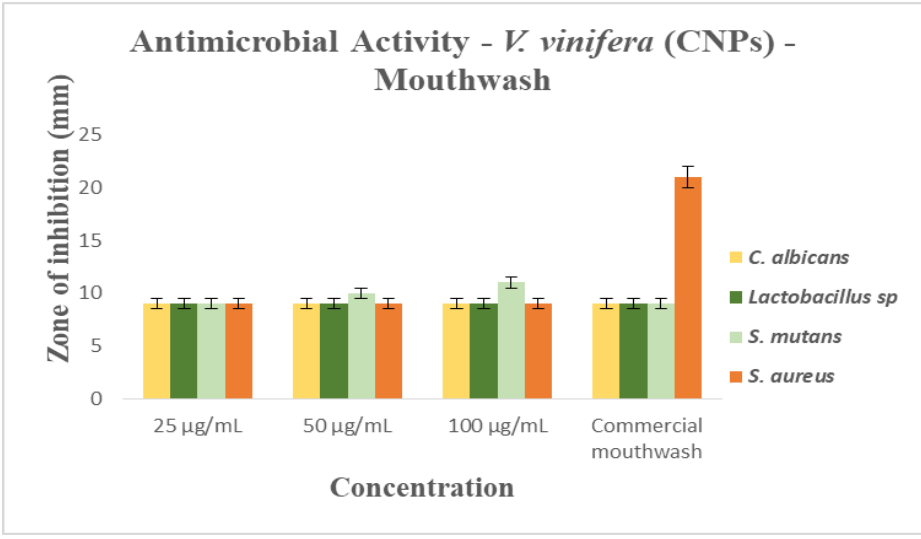


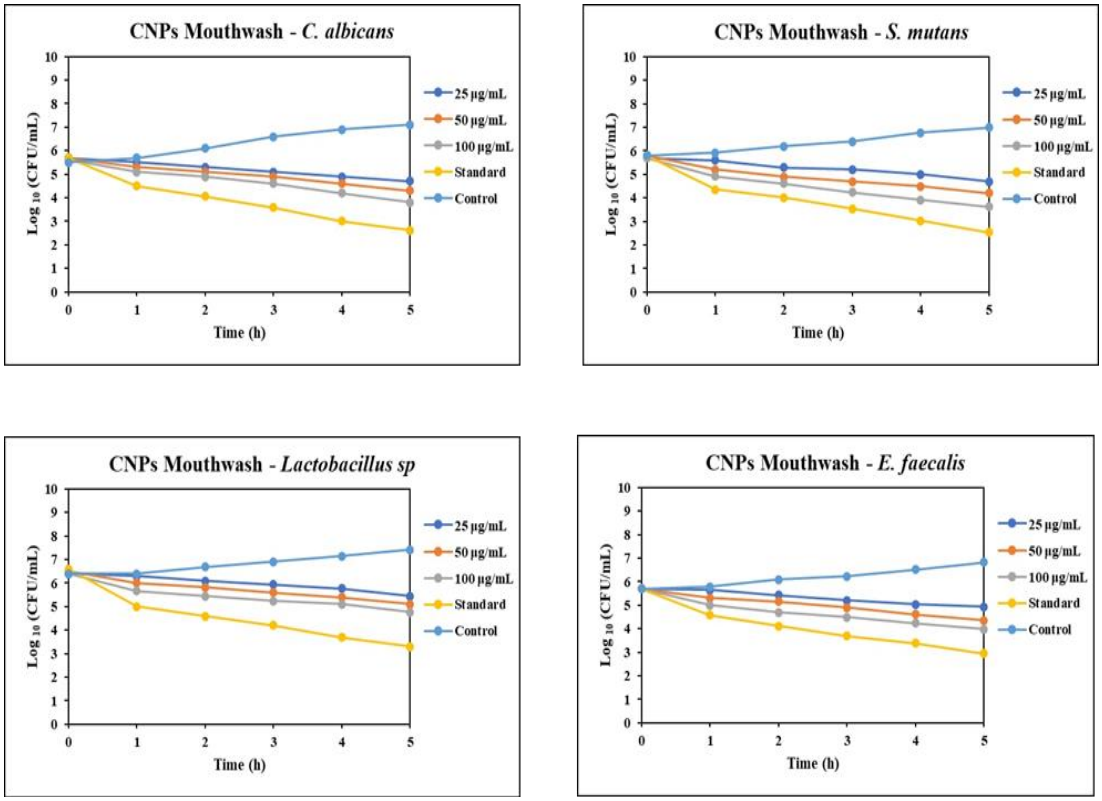
Figure 3: Displays antibacterial effectiveness of carbon nanoparticles compared to commercial mouthwash is tested against various pathogens.



The antimicrobial property of chitosan-incorporated Vitis vinifera-mediated carbon nanocomposite is tested by agar well diffusion method against four pathogens. In Figure 2 we

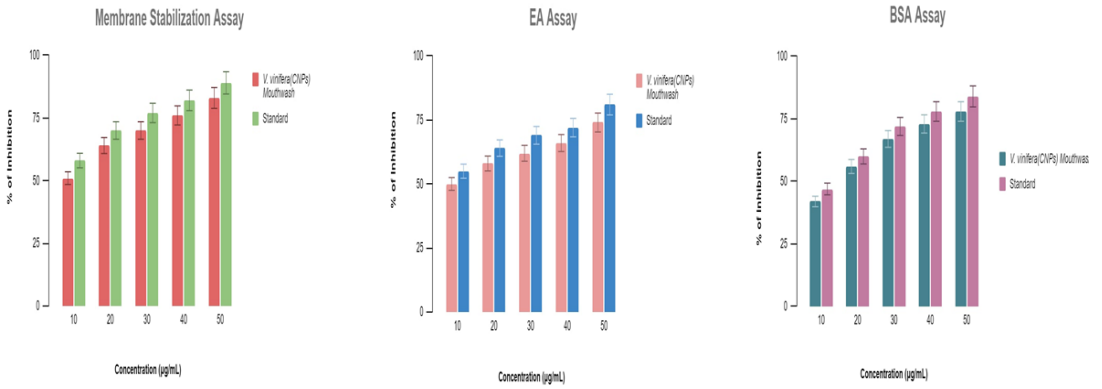
can see the visual representation of the result of the antibacterial activity of nanoparticles. In the Figure 3, we can see the graphical representation of the antibacterial activity of carbon nanoparticles against dental pathogens. The largest zone of inhibition against S. aureus is given by commercial mouthwash (21nm). The biggest zone of inhibition against S. mutans is given by nanoparticles at a concentration of 100µg/ml. The zone of inhibition against C. albicans and Lactobacillus sp is the same for carbon nanoparticles and commercial mouthwash.

Figure 4: Time kills curve analysis of oral pathogens including, C. albicans, S. mutans, E. faecalis, and Lactobacillus sp.



The antimicrobial effect of carbon nanoparticles is assessed by time-kill curve assay against oral pathogens C. albicans, S. mutans, Lactobacillus sp, and E. faecalis and compared it with control and standard. At all concentrations of carbon nanocomposites, a noticeable decrease in growth is seen. Control doesn't have any effect in reducing the growth of pathogens as it only consists of broth culture. Carbon nanocomposites' effectiveness increased with an increase in concentrations. At 25µg/ml carbon nanoparticles exhibited the least effect followed by 50µg/ml and at 100µg/ml they exhibited the highest effect. The standard decreased growth of pathogens was observed at the highest concentration 100µg/ml. The carbon nanocomposites at the concentration of 100µg/ml exhibited a great decrease in growth analyzed using an ELISA reader and noted. In which, E. faecalis and S. mutans growth was reduced by the synthesized carbon nanocomposites, in comparison, Lactobacillus sp. and C. albicans were little sensitive to the sample as they showed similar reduction of growth.

Figure 5: Anti-inflammatory activity of Vitis vinifera – carbon nanoparticles mouthwash was represented graphically by Membrane stabilization assay, EA assay, and BSA assay.



The anti-inflammatory effect of carbon nanoparticles is assessed by BSA assay, EA assay, and Membrane stabilization assay. The percentage of inhibition of carbon nanocomposites at several concentrations (10,20,30,40,50µg/ml) is compared with the standard. Carbon nanoparticles exhibited dose-dependent effects. The maximum effect shown by carbon nanoparticles at 50µg/ml is 78% and the standard is observed as 84% in bovine serum albumin assay. In the egg albumin denaturation assay, the carbon nanocomposites exhibited 76% at the concentration of 50 µg/ml, 68% at 40 µg/ml, 64% at 30 µg/ml, 59% at 20 µg/ml, 48% at 10 µg/ml. The standard shows that 81% of the concentration of 50 µg/ml, 72% at 40 µg/ml, 69% at 30 µg/ml, 64% at 20 µg/ml, and 55% at 10 µg/ml. In MS assay, the synthesized carbon nanocomposites exhibit 83% at the concentration of 50 µg/ml, 76% at 40 µg/ml, 72% at 30 µg/ml, 61% at 20 µg/ml, 51% at 10 µg/ml. The standard shows that 89% of the concentration of 50 µg/ml, 82% at 40 µg/ml, 77% at 30 µg/ml, 70% at 20 µg/ml, and 58% at 10 µg/ml. In conclusion, the anti-inflammatory of synthesized carbon nanocomposites is exhibited based on a dose-dependent manner, which is graphically represented in Figure 5.

4. Discussion:

The synthesis of Vitis vinifera carbon nanoparticle and mouthwash was successful. Its antimicrobial and anti-inflammatory activity was evaluated to check its effectiveness. The antimicrobial property of Vitis vinifera carbon nanoparticles is evident through agar well diffusion and time kill curve assays. They showed concentration-dependent antimicrobial activity. At higher concentrations of nanoparticles, there was more reduction in bacterial count. The Vitis vinifera carbon nanoparticle was more effective against Lactobacillus sp followed by C. albicans, S. mutans, and S. aureus. The anti-inflammatory property of Vitis vinifera carbon nanoparticles is evident through Egg albumin assay, Bovine Serum Albumin assay, and membrane stabilization assay. They showed concentration-dependent anti-inflammatory activity. Standard performed better than nanoparticles. In the previous study conducted by Waseem et al., the antimicrobial effectiveness of pomegranate peel carbon nanoparticles was studied using the agar diffusion method. It was studied against four

pathogens Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Escherichia. They tested the antimicrobial effects of CNPs at various concentrations and compared it with standard (gentamycin). They showed concentration-dependent antimicrobial effects. The standard performed better than carbon nanoparticles (Parmar et al., 2018). In the study conducted by Jimin Long et al., the anti-inflammatory properties of multi-walled carbon tubes are studied. They showed potent anti-inflammatory effects (Qureshi et al., 2021). In the study conducted by Zheng Qu et al., carbon dots are synthesized and their anti-inflammatory properties are assessed. Carbon dots exhibited good anti-inflammatory activity ((Long et al., 2018)). In a previous study conducted by Xiaoke Wang et al., the anti-inflammatory ability of carbon dots derived from silkworm cocoons was evaluated and they exhibited anti-inflammatory activity (Qu et al., 2020).

5. Conclusion:

From the above study, we could conclude we successfully prepared mouthwash from carbon nanoparticles synthesized from Vitis vinifera. Mouthwash prepared from carbon nanoparticles synthesized from Vitis vinifera exhibited dose-dependent antimicrobial activity. It is evident through agar well diffusion and time-kill curve assay. Its anti-inflammatory property is also evident through BSA assay, EA assay, and membrane stabilization assay. This mouthwash can be used as an alternative to traditional mouthwashes that are available in the market because of fewer effects. Further studies can be conducted to increase its effectiveness,

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