

ANTI-INFLAMMATORY AND CYTOTOXIC EFFECT OF STEVIA AND NEEM BASED HERBAL FORMULATION

Running title: Anti-inflammatory and cytotoxic effect of stevia and neem

ABSTRACT

Introduction: Nanotechnology is being used in developing countries to treat diseases and prevent health issues. Stevia, conjointly stated as *Stevia rebaudiana* member of the chrysanthemum family, a subgroup of the Asteraceae family. *Azadirachta indica* is a native tree to Asian nations. *Azadirachta indica* could facilitate the fight against plaque buildup and stop periodontal disease. Applied science in drugs needs the employment of nanoparticles to transmit the drug, heat, lightweight, or alternative substances to specific cell sorts like cancer cells. **Aim:** In this present investigation, *Stevia and Neem-based* plant extract was prepared and analyzed its anti-inflammatory and cytotoxic effects. **Materials and methods:** The plant extract was prepared and an anti-inflammatory and cytotoxic effect was done using a UV-Beckmann spectrometer. The anti-inflammatory activity of nanoparticles prepared using plant extract was investigated by albumin denaturation assay. The results of the test were described as the standard deviation and analyzed using UV-Vis spectroscopy. For the cytotoxic activity, wells are used, wherein the mortality rate of the shrimps was estimated when the plant extract mediated of nanoparticles of different concentrations was added. **Result:** The nanoparticles synthesized using the stevia and neem extract showed the highest absorbance at a concentration of 50 μ L (90%) when subjected to inhibition of albumin denaturation assay to check for its anti-inflammatory activity. Brine shrimp lethality was done and the cytotoxicity of these nanoparticles was found to be increasing with increasing concentration of the administered nanoparticles. **Conclusion:** Anti-inflammatory and cytotoxic activity of Neem and Stevia mediated nanoparticles showed a successful outcome in both the assays.

Keywords: Neem, Stevia, Nanotechnology, Anti-inflammatory, Cytotoxicity effect, mouth wash, Innovative technique.

INTRODUCTION

Nanotechnology has been introduced in our daily routine. This technology has been applied in multiple fields through an integrated approach. An increasing number of applications and products containing nanomaterials or a minimum of nano-based claims became available. This also happens in pharmaceutical research (1). Nanotechnology has been widely used in synthesizing drugs in the form of nanoparticles, nanocapsules, nanospheres and nanoemulsions. The employment of nanotechnology within the development of the latest medicines is now a part of our research and within the international organization (EU) it's been recognized as a Key Enabling Technology, capable of providing new and innovative medical solutions to deal with unmet medical needs. The administration of nanotechnology for medical purposes has been termed nanomedicine and is defined because of the use of nanomaterials for the control, prevention, and treatment of diseases. However, the definition of nanomaterial has been controversial among the assorted scientific and international regulatory corporations. These properties greatly increase a group of opportunities within drug development; however, some concerns about issues of safety have emerged (2). The physicochemical properties of the nanoformulation which might source the alteration of the pharmacokinetics, namely the absorption, distribution, elimination, and metabolism, the potential for more easily cross biological barriers, toxic properties, and their endurance within the environment and form are some samples of the concerns over the apparatus of the nanomaterials.

Stevia, conjointly stated as *Stevia rebaudiana* member of the chrysanthemum family, a subgroup of the Asteraceae family (3). Some are usually referred to as angiospermous yellowwood stevia, honey leaf plant, sweet chrysanthemum, sugar leaf, etc. It is native to northeast South American countries, Brazil and Argentina. It is currently grown in alternative elements of the planet, together with North American countries and a district of Asia and Europe. Most likely best stated as a supply of natural sweeteners (4). It is used as a non-nutritive sweetener and flavorer supplement. A non-nutritive sweetener carries very little to no calories. *Stevia* is utilized as a healthful variety of sugar in several meals and beverages. *Stevia* will act as a wonderful substitute for sugar. *Stevia* leaves are roughly forty times sweeter than processed sugar (2,5,6). This sweetness is attributable to a variety of organic compounds together with stevioside, steviolbioside, rebaudiosides A-E, and dulcoside. Since the demand for low-calorie food alternatives is high and polygenic disorder is on the rise, stevia has drawn the attention of health-conscious people. Stevioside might be a non-carbohydrate organic compound. Hence, it lacks the properties that plant products and alternatives to carbohydrates (7). *Stevia* extracts, like rebaudioside-A, are found to be three hundred times

sweeter than sugar. Besides being a near-zero calorie food ingredient, *Stevia* has distinctive properties like high-temperature tolerance and non-fermentativeness. *Stevia* is going to be used orally for medical functions like lowering vital signs, treating polygenic disorder, heartburn, high acid levels inside the blood, weight loss, stimulating the guts rate, and water retention. Being a non-carbohydrate sweetener, stevia would not favor the enlargement of microorganism mutans bacteria inside the mouth that is attributed to be an inductive agent of decay and tooth cavities (8,9). On the alternative hand, bound compounds in *Stevia* are rather found to inhibit caries-causing bacteria inside the mouth. *Azadirachta Indica* is a native tree to Asian nations (10). *Azadirachta indica* leaves contain over a hundred thirty different types of biological compounds, like Nimbin, nimandial that facilitate heal the body and promote healthy living (11). *Azadirachta indica* leaves are used in treating microorganism infections including chickenpox. *Azadirachta indica* is additionally presupposed to cut back inflammation, improve liver health, alleviate pain, preserve sight, stimulate the system, and shield against heart diseases (12). *Azadirachta indica* could facilitate the fight against plaque buildup and stop periodontal disease. Applied science in drugs needs the employment of nanoparticles to transmit the drug, heat, lightweight, or alternative substances to specific cell sorts like cancer cells (13).

Folk medicine as an anti-inflammatory agent against pain, fever, arthritis and migraine. Inflammation sometimes happens once infectious microorganisms like bacteria, viruses, or fungi invade the body, reside specifically in tissues, and flow into the blood. Inflammation happens in response to processes like tissue injury, cell death, cancer, anemia, and degeneration (14). Mostly, each innate response nonetheless as a result of the accommodative immune reaction is concerned with the formation of inflammation (15). The cytotoxic activity of ABCs toxins resides inside the hypervariable CCTR domain of the C element. Genetic proof indicates that the variable C-terminal domains are doubtless to have evolved severally of the N-terminal rhs domains (16). Conjointly, their sequences are numerous but share similarity with a variety of varied supermolecule folds, several of which are involved in cytotoxic pathways (17,18).

The previous study involves the synthesis of selenium nanoparticles incorporating garlic oil. It can be deduced that garlic oil-mediated selenium nanoparticles have a good cytotoxicity activity at high concentrations (19). In another study, the green synthesis of selenium nanoparticles was easy and simple. The study concluded that coriander Oleoresin mediated selenium nanoparticles had good anti-inflammatory activity (20). The cytotoxicity assessment was carried out using a brine

shrimp lethality assay. The iron nanoparticles did not show any cytotoxicity to the brine shrimp. It can be used to find new drugs in the future against many diseases. In dentistry, it can be used as a mouthwash, toothpaste. This eco-friendly synthesis from plant extract has to be inexpensive, convenient and can be safely used in a wide range of medical and dental fields. Our team has extensive knowledge and research experience that has translate into high quality publications (21–25),(26),(27),(28),(29),(30),(31-33),(34–40) In this present investigation, *Stevia and Neem-based* plant extract was prepared and analyzed for its anti-inflammatory and cytotoxic effects.

MATERIALS AND METHOD

Preparation of plant extract

Fresh *Stevia and Neem* extract powder was purchased from the herbal health care center. 2.5gm of neem and stevia was added to 100ml of distilled water to the beaker and boiled for 10-20 minutes in the heating mantle. The boiled extract was filtered using a filter paper (figure 1).



Figure 1: Image A representing 2.5gm of Stevia was measured under the weighing machine. Image B representing 2.5gm of Neem was measured under the weighing machine. Image C representing the Neem and Stevia aqueous formulation and Image D representing aqueous extract of Neem and Stevia boiled for 10-20 minutes at 60 degree Celsius.

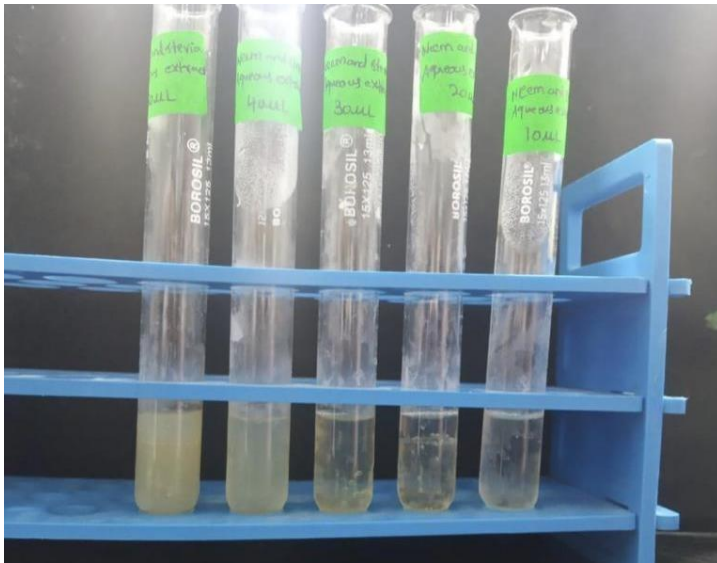
Anti-inflammatory activity

The anti-inflammatory activity for *Stevia and Neem* was tested by the following convention proposed by Mizushima and Kobayashi with specific alterations (Pratik Das et al., 2019). 0.05 mL of *Stevia and Neem* of various fixation (10 μ L,20 μ L,30 μ L,40 μ L,50 μ L) was added to 0.45 ml bovine serum albumin (1% aqueous solution) and the pH of the mixture was acclimated to 6.3 utilizing a modest quantity of 1N hydrochloric acid. Care should be taken while micro-pipetting to

avoid bias, standard values which were obtained prior are used for comparison. These samples were incubated at room temperature for 20 min and then heated at 55 °C in a water bath for 30 min. The samples were cooled and the absorbance was estimated spectrophotometrically at 660 nm. Diclofenac Sodium was used as the standard. DMSO is utilized as a control (figure 2).

Percentage of protein denaturation was determined utilizing the following equation,

$$\% \text{ inhibition} = \frac{\text{The absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$



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Figure 2: Image showing the Preparation of anti-inflammatory activity assessed using albumin denaturation assay of the reaction mixture at different concentrations compared to a positive control diclofenac sodium.

Cytotoxic activity

Brine Shrimp Lethality Assay:

Saltwater preparation: 2g of iodine-free salt was weighed and dissolved in 200ml of distilled water. 6 well ELISA plates were taken and 10-12 ml of saline water was filled. To that 10 nauplii were slowly added to each well (20 μ L,40 μ L,60 μ L,80 μ L,100 μ L). Then the nanoparticles were added according to the concentration level. The plates were incubated for 24 hours. After 24 hours, the ELISA plates were observed and noted for the number of live nauplii present (figure 3) and calculated by using the following formula,

Number of dead nauplii /number of dead nauplii+number of live nauplii \times 100o

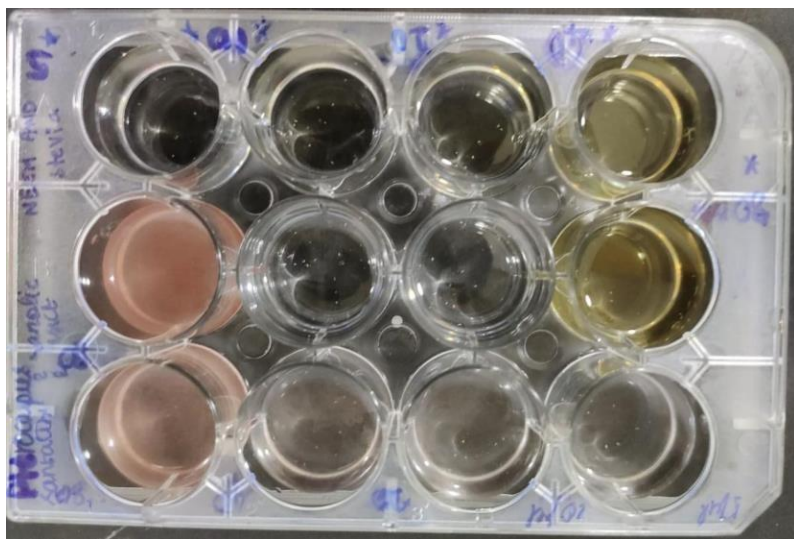


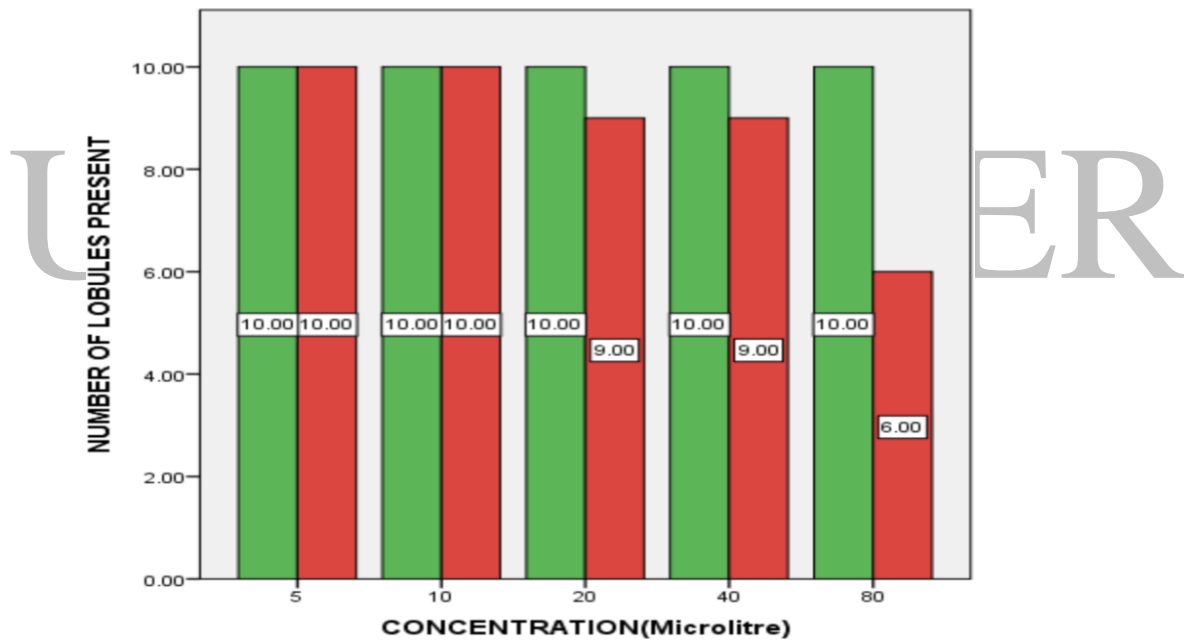
Figure 3: Image showing the preparation of cytotoxic activity using neem and stevia mediated nanoparticles with different concentrations of cytotoxic effect.

The procedure was carried out by the random sampling method. Validation of the procedure was done by nano experts followed by Correlation analysis of the results using SPSS software. Only anti-inflammatory and cytotoxic effects were done. Further study will be done on antidiabetic and antimicrobial activities.

RESULT AND DISCUSSION

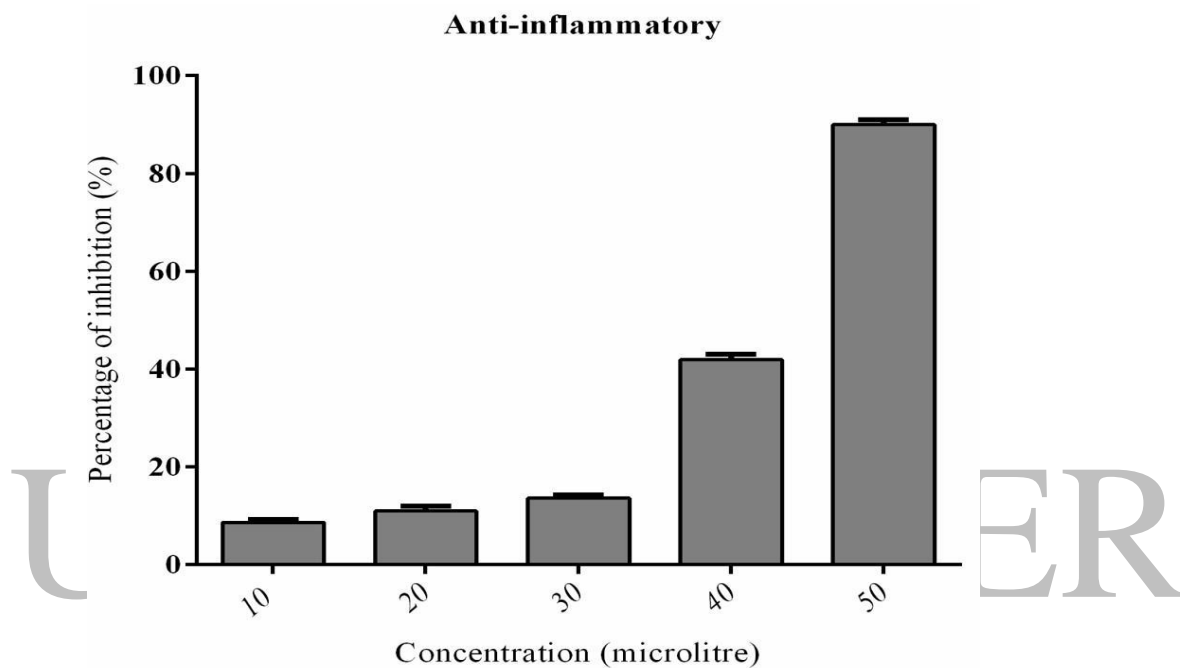
Anti-Inflammatory activity

Figure 4: Graph showing the anti-inflammatory activity of Neem and Stevia mediated nanoparticles. The spectrophotometric absorbance value and five different concentrations of the extract are represented. The X-axis shows concentration, the Y-axis shows percentage of inhibition. The graph shows positive correlation with rise in concentration.



Cytotoxic activity

Figure 5: The graph represents the cytotoxicity effect of the plant extract. The X-axis represents different concentrations and the Y-axis represents the number of lobules decrease with an increase concentration



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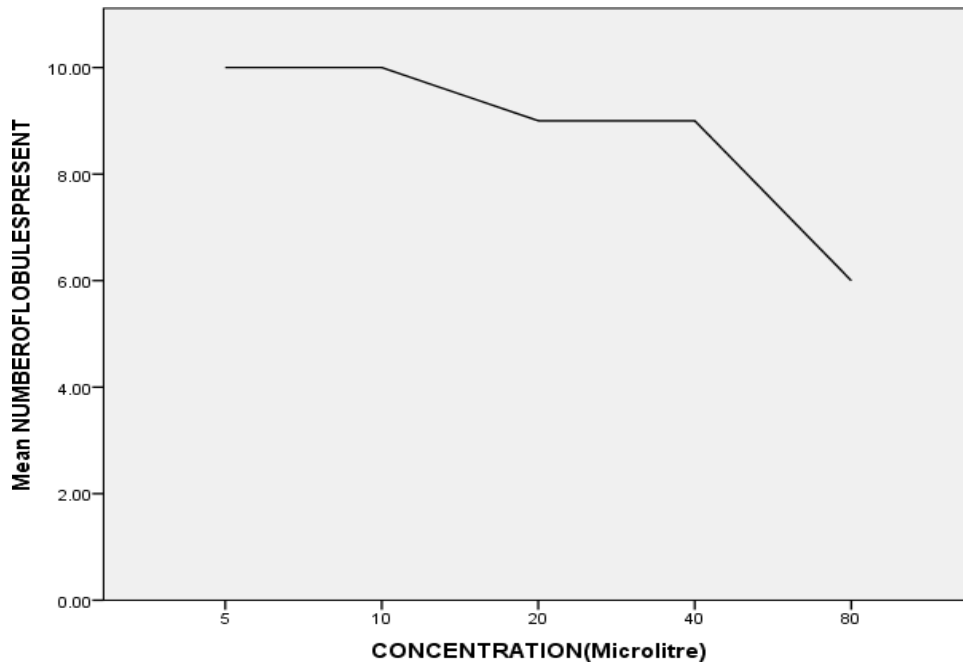


Figure 6: The given line diagram represents the cytotoxic effect of plant extract. The X-axis represents the different concentrations in Microlitre and the Y-axis represents the mean number of lobules present.

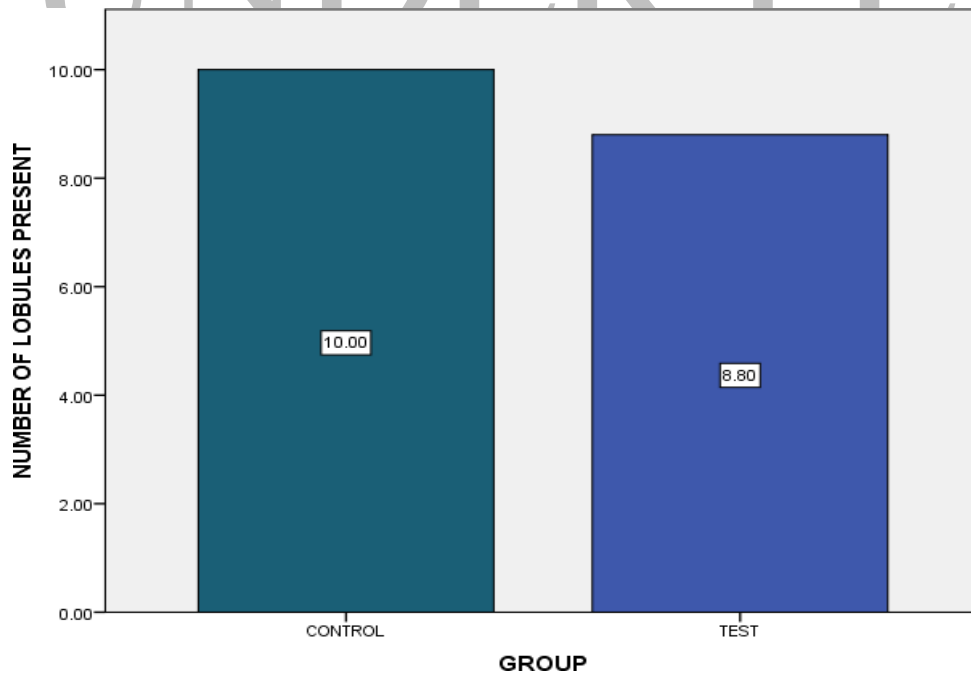


Figure 7: The given bar graph represents the total mean value of the cytotoxic effect of plant extract. The X-axis represents the group and Y-axis represents the number of lobules present.

Estimation of Anti-inflammatory activity

The anti-inflammatory activity was estimated in five different concentrations of the reaction mixture (10 μ L, 20 μ L, 30 μ L, 40 μ L, 50 μ L). Anti-inflammatory activity at different concentrations

from 10 μ L to 50 μ L showed (figure 4) absorbance at 9%, 12%, 14%, 41% and 91% respectively. Standard showed an 83% inhibition. Plant extract mediated by nanoparticles at 50 μ L of concentration exhibited the highest anti-inflammatory activity when compared to standard diclofenac sodium.

Cytotoxicity Analysis

Brine shrimp lethality assay is an important test in the study of toxicity that gives us information about the cytotoxic effect exhibited by a bioactive compound to cells. The viability of the nauplii was analyzed for different concentrations of nanoparticles that are synthesized from *Stevia and Neem*. After 24 hours, it was found that at a minimal concentration of 5 μ L and 10 μ L 10 numbers of the nauplii were alive, at 20 μ L and 40 μ L nine nauplii were alive and at 80 μ L six nauplii were alive. Whereas the control showed 10 numbers of the nauplii to be alive (figure 5).

From the result, it is concluded that the combination of Neem and Stevia possesses greater anti-inflammatory activity than an individual plant extract. The anti-inflammatory activity was approximated by in vitro screening methods like protein denaturation and the HRBC method. Denaturation of proteins is a well-documented source of inflammation. There was a gradual increase in the anti-inflammatory activity of neem and stevia with an increase in the concentration of neem and stevia and was greater when compared to the standard diclofenac sodium at a higher concentration. In another study selenium nanoparticles of Capparis decidua was described as an anti-inflammatory activity at 50 μ L of concentration shows the very best anti-inflammatory activity at 87% inhibition (41). The present study indicated that the biosynthesis of nanoparticles can be made possible from Neem and Stevia and possess fewer cytotoxic effects. The high lethality of the brine shrimps caused by Stevia and Neem leaf is indicative of the presence of potent cytotoxic components. Azadirachtin that is derived from the neem tree is both a toxicant and antifoliant which affects brine shrimp lethality. In another study, the cytotoxic assessment was carried out using a brine shrimp lethality assay (20). The iron nanoparticles of the cytotoxicity to the brine shrimp did not show any effect. Nanoparticles were successfully synthesized using plant extract. Determining the various applications of nanoparticles synthesized from plant extract can be utilized in the dentistry and the medical field.

Denaturation of tissue protein is one of the well-known causes of inflammatory and arthritic diseases. Agents that can stop protein denaturation, therefore, can be used as anti-inflammatory drug development. This broader inhibitory activity may be beneficial for managing excessive

inflammation, which is generally triggered through multiple defense pathways present in the immune system (42). The most common diseases of tooth-supporting structures are plaque-induced inflammatory alteration in the gingiva and the periodontium. It has been demonstrated that periodontal diseases are caused by bacterial infection (43). Herbal medicines, derived from botanical sources, are applied in dentistry for an extended history to inhibit microorganisms, reduce inflammation, soothe irritation, and relieve pain. It was recently disclosed that a significant number of herbal mouthwashes have achieved promising ends up in plaque and gingivitis control. Herbal mouthwashes are manufactured with extracts and essential oils of phytotherapeutic plants, owning a mixture of active agents such as catechins, tannins, and sterols. The combination of natural compounds inside the herb- or plant-derived materials generally performs gentle remedial effects. In contrast with the antimicrobial mechanisms by synthetic chemicals, herbal mouthwashes can have additional anti-inflammatory and antioxidant properties, which could further benefit gingival health. This study could be continued using more volume of samples and this can be done under in vivo study in the near future.

CONCLUSION

Within the limitations of the study, we conclude that nanoparticles on Stevia and Neem extract have considerable anti-inflammatory activity and cytotoxic effect. It showed a successful outcome in the performed assays. Hence it can be used in finding new drugs with higher potency and lesser toxicity. In dentistry, it can be used as a mouthwash to prevent plaque, periodontal disease, and dental caries.

ACKNOWLEDGEMENT:

The authors are thankful to Saveetha Institute of Medical and Technical Sciences, Saveetha Dental College and Hospitals, Saveetha University for giving a platform to conduct the study.

CONFLICT OF INTEREST:

The authors would like to declare no conflict of interest in the present study.

FUNDING:

The present project is supported by:

- Selvam Maligai, Dharmapuri District
- Saveetha Dental College and Hospitals,

- Saveetha Institute of Medical and Technical Sciences,
- Saveetha University

AUTHORS' CONTRIBUTIONS

'S. Selvapriya' designed the study, performed the methods and wrote the first draft of the manuscript and 'Dr. Priyadarshini R' performed the statistical analysis and wrote the protocol, 'Dr. Rajeshkumar S' managed the analyses of the study and managed the literature searches and "Dr. Palati Sinduja" managed the final drafting and editing of the manuscript. All authors read and approved the final manuscript.

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