ORIGINAL RESEARCH

Evaluation of Cytotoxicity of *Allium sativum* (Garlic Extract) against Human Dental Pulp Fibroblasts

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Abstract

Background: Vital pulp therapy procedures in primary dentition focuses on preservation and maintenance of pulp tissue that has been compromised due to caries, trauma, etc. Several pulp dressing materials have been used in primary teeth and some natural materials from the field of traditional medicine have also been introduced as medicaments in vital pulp therapy. The understanding of biologic and cytotoxic properties of newer materials is important for safe clinical usage. The biologic compatibility of these newer materials is imperative to limit or avoid tissue irritation or degeneration.

Aim: To evaluate the cytotoxic effects of *Allium sativum* on cultured human primary dental pulp fibroblasts.

Materials and methods: Primary pulp fibroblasts were cultured from the pulp tissue obtained from extracted deciduous primary canines and central incisor teeth. The freshly prepared concentrations of 1000, 500, 250, 125, and 62.5 µg/mL *A. sativum* extract were added to the 96-well plate in triplicates to which culture medium containing fourth passage cell suspension was added previously. Cells without treatment served as control, while cells treated with 5% dimethyl sulfoxide (DMSO) served as toxic control. After the addition of experimental and control agents, cells were incubated for 24 and 48 hours at 37°C in 5% CO_2 atmosphere. After the incubation period, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the number of viable cells. Absorbance was read with a microplate reader at 570 nm wavelength and the relative viability of dental pulp fibroblasts at various concentrations was expressed as color intensity of the experimental wells relative to that of control. The percentage of cell viability was also calculated accordingly.

Results: The MTT assay results revealed that *A. sativum* extract, in all the concentrations tested at both the time intervals maintained a cell viability of greater than 90%. At 24 hours, the mean absorbance value of untreated control wells was recorded as 0.84400 ± 0.00916 with 100% cell viability. Among all the concentrations of garlic extract tested, highest mean absorbance value of 0.83933 ± 0.00550 with 99.44% cell viability was recorded for 62.5 µg/mL concentration. At 48 hours, the mean absorbance value of untreated control wells was recorded as 1.22767 ± 0.01106 with 100% cell viability, and the highest mean absorbance value of 1.22567 ± 0.01006 with 99.83% cell viability was recorded for 62.5 µg/mL concentration. The cell viability did not seem to be affected by the concentration of *A. sativum* extract at 24 hours. However, at 48 hours, the sensitivity of the cells was observed to be dependent on the concentration of *A. sativum* with a decrease in the viability of cells noted with the increase in concentration.

Conclusion: *A. sativum* extract is noncytotoxic in nature and preserves the vitality of cultured human primary dental pulp fibroblasts making it a suitable material for use in vital pulp therapy procedures of primary teeth.

Keywords: *Allium sativum*, Cytotoxicity, Garlic extract, Human dental pulp fibroblasts.

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Introduction

The main aim of pediatric pulp therapy is to support the health and integrity of the teeth and supporting structures in children. Vital pulp therapy procedures focus on preservation and maintenance of vitality of pulp compromised due to caries, trauma, etc. At present, there are distinctive strategies and ways for the treatment of pulp relying on the damage extension and pathologic pulp involvement.[1] Pulpotomy is a conservative therapeutic alternative involving the excision of infected coronal pulp and dressing the noninfected pulp with a biocompatible material. The objective is that radicular pulp tissue should remain vital and healthy without any unfavorable clinical signs and/or symptoms.[2]

Several medicaments have been tried for capping the radicular portion of pulp after the amputation of infected coronal pulp. An optimum material or medicament must have favorable physical and biological traits such as protecting the tissues from bacterial infection, being biocompatible, reducing inflammation and inducing cell proliferation to aid in the reconstruction of damaged tissue.[3] Several materials such as formocresol,[4] glutaraldehyde,[5] ferric sulfate,[6] collagen material,[7] mineral trioxide aggregate,[8,9] and Biodentine[10] were used as pulp dressing materials in primary teeth. However, a continuous exploration for a more suitable and biocompatible material is on as each of the above materials have been associated with advantages as well as disadvantages.

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Some naturally occurring materials from the domain of traditional medicine have also been tried as medicaments in vital pulp therapy as an auxiliary to commercially obtainable artificial products. One such medicine is *Allium sativum*, commonly known as garlic.[11] Garlic has been reported to exhibit antimicrobial[12] and antioxidant effects,[13] regulate the immune system[14] and contribute to wound healing.[15] Garlic is also found to be effective against a wide range of gram-negative and gram-positive bacteria.[12]

Extracts of *A. sativum* have been used for varying applications in dentistry.

Elheeny[16] used *A. sativum* extract as an intracanal irrigant for pulpectomy procedure of primary molars and evaluated its efficacy both clinically and radiographically. Prabhakaran et al.[17] and Koppolu et al.[18] evaluated the smear layer removal capacity of garlic extract in permanent root canals. Eswar et al.[19] demonstrated the efficacy of garlic extract when used as an intracanal medicament in premolar teeth contaminated with *Enterococcus faecalis*. Groppo et al.[20] tested the antimicrobial efficacy of two crude garlic extracts against oral microorganism when used as 1-minute mouthwash. Mohammad et al.[21] and Kahvand et al.[22] used *A. sativum* oil as a medicament for vital pulp treatment of primary teeth and tested its efficacy clinically and radiographically in comparison with formocresol. Both studies reported favorable results with *A. sativum* and recommended it as a pulpotomy medicament for primary teeth.

The understanding of biologic and cytotoxic properties of newer materials is important for safe clinical usage.[23] The biologic compatibility of these newer materials is imperative to limit or avoid tissue irritation or degeneration. *In vitro* cytotoxicity assays help in measuring the response of cells against a drug, material, or an extract.[24] Primary cell cultures, that is, pulp fibroblasts derived from human dental pulp of primary teeth are appropriate for testing biocompatibility of materials used for vital primary pulp therapy.[25]

Since there are no reported studies evaluating the cytotoxic effects of *A. sativum* on human primary dental pulp, the purpose of the present study was to evaluate the cytotoxicity of *A. sativum* on cultured human primary dental pulp fibroblasts.

Materials and Methods

Source of Primary Pulp Tissue

Primary pulp tissue was obtained from two healthy deciduous canines indicated for orthodontic extraction and from one retained primary central incisor tooth after obtaining parental informed consent. Ethical clearance for the study was obtained from the Institutional Review Board and Ethical Committee (SRGCDS/2019/629).

After extraction of teeth, pulp tissue was carefully extirpated from the teeth with sharp spoon excavator and sterile broaches. The primary pulp tissue thus obtained was washed in phosphate buffered saline (PBS) for 2–3 seconds, placed in Dulbecco's Modified Eagles Medium (DMEM) and transported to the lab in aseptic conditions under 5°C for culturing pulp fibroblasts.

Isolation and Culture of Human Primary Dental Pulp Fibroblasts

The pulp tissue was placed in a sterile Petri dish and cut into small pieces of about 1 mm and fat tissue was removed. The tissue was subjected to enzymatic digestion with Dulbecco's Modified Eagles Media containing 5 mg/mL of Dispase and 3 mg/mL Collagenase II/IV at 4°C for 2–4 hours. The isolated tissue fragments were incubated for 15–20 minutes at room temperature in 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) with intermittent shaking. Trypsin enzyme helps in detachment of cells from the surface of the glass or plate. Trypsin was then inactivated by adding 4 mL of DMEM with 10% fetal bovine serum (FBS). The trypsinized tissue was filtered through 40 μm filter to obtain single-cell suspension containing cells. The cells were centrifuged at 1200 rpm for 10 minutes and the pellet of cells was resuspended in 1 mL PBS containing 2% FBS.

The cell suspension thus obtained was cultured in a medium of DMEM with 10% FBS containing 1% antibiotic–antimycotic solution in a humidified incubator at 37°C with 5% CO_2. Maintenance of cell growth was monitored everyday by changing the medium once in 3 days until the cells reached 70–80% confluence. The cells were then subcultured into two flasks by adding 1 mL of 0.25% trypsin-EDTA and incubated for 5 minutes for detachment of cells. Once the cells were detached, tryspin-EDTA was diluted with 2 mL of DMEM to inactivate the action of trypsin. The cells were then centrifuged at 1800 rpm for 5 minutes and pellet of cells was collected by discarding the supernatant solution.

To the pellet containing cells, 1 mL of DMEM was added, mixed well and subcultured till the fourth passage cells were obtained. After reaching fourth passage, cells were then harvested by trypsinization and concentration of the cells was counted by hemocytometry using an inverted binocular biological microscope. Based on the obtained concentration of cells, the cells were diluted with media as per requirement for 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Preparation of *A. sativum* Extract (Garlic Extract)

Fresh and healthy garlic cloves (250 gm) were purchased from the local market. The cloves were cleaned, peeled, and rinsed in 70% absolute ethanol for 60 seconds to ensure sterility. By using sterile mortar and pestle, cloves were finely homogenized to make a paste. The finely homogenized paste of 200 gm was transferred to a conical flask and was dissolved in 300 mL of 70% absolute ethanol. The contents of the conical flask were thoroughly dissolved using magnetic stirrer for 48 hours. Afterward, the dissolved solution was filtered through a fresh filter paper with the help of funnel. The filtered contents were transferred to sterile Petri plates and were allowed to dry for 2–4 days at room temperature. Once the extract became paste, it was scraped and collected into vials. This crude extract (approximately, 48 gm) was stored at 4°C for further use. Further on, various concentrations of garlic extract were made by serial dilution of crude extract with DMEM.

Cytotoxicity Assay

About 200 μL of cell suspension was seeded at required cell density (20,000 cells per well), without the test agent in a 96 well plate and the cells were allowed to grow for about 24 hours. *A. sativum* extract in concentrations of 1000, 500, 250, 125, and 62.5 μg/mL were added to the wells, respectively in triplicates. Cells without treatment served as control. Cells treated with 5% dimethyl sulfoxide (DMSO) served as toxic control. After addition of the experimental and controls reagents, plates were incubated for 24 and 48 hours at 37°C in a 5% CO_2 atmosphere. After the incubation period, the plates were removed from the incubator, spent media was removed and were subjected to MTT assay to determine the number of viable cells.

3-(4,5-dimethyl-2-thiazolyl)-2,5-Diphenyltetrazolium Bromide Assay

After the incubation period of 24 and 48 hours, on removal of spent media, MTT reagent was added to a final concentration of 0.5 mg/mL of total volume. The plate was then wrapped with aluminum foil to avoid light exposure and incubated further for 3 hours (mitochondrial dehydrogenase produced by viable cells reduces yellow MTT dye into insoluble blue formazan crystals). After the incubation period, overlying MTT reagent was cleared, 100 µL of solubilizing solution (DMSO) was added and gently stirred to enhance the dissolution. Occasional pipetting was done to completely dissolve the MTT formazan crystals especially in dense cultures. Absorbance was read with a microplate reader at 570 nm wavelength and the relative viability of dental pulp fibroblasts at various concentrations was expressed as color intensity of the number of experimental wells relative to that of control. The percentage of viable cells were calculated by using the formula:

$$\%Cell\ viability = \left(\begin{matrix} Mean\ absorbance\ of\ treated\ cells\ / \\ Mean\ absorbance\ of\ untreated\ cells \end{matrix} \right) \times 100$$

Statistical Analysis

Descriptive and analytical statistics were done using Statistical Package for Social Sciences (SPSS) Version 24.0 (IBM Corporation, Chicago, United States). The mean absorbance values in different groups were compared using one-way analysis of variance. Pairwise comparison between the groups was carried out using *post hoc* Tukey test. The level of significance was set at $p < 0.05$.

Results

The mean absorbance values of primary pulp fibroblasts exposed to various concentrations of *A. sativum* extracts including the control groups (untreated and toxic controls) at 24 and 48 hours are depicted in Table 1 and 2, respectively. The mean absorbance value calculated is the average of recordings of three wells at any given time interval. Greater the mean absorbance value, higher is the cell survival. The percentage of cell viability calculated (as per the formula mentioned previously) for each of the groups at 24 and 48 hours is also depicted in Table 1 and 2, respectively.

Cytotoxicity of Various Concentrations of *A. sativum* Extract on Primary Pulp Fibroblasts at 24 Hours

The mean absorbance value of untreated control wells was recorded as 0.84400 ± 0.00916 with 100% cell viability at 24 hours. Among all the concentrations of garlic extract tested, highest mean absorbance value of 0.83933 ± 0.00550 with 99.44% cell viability was recorded for 62.5 µg/mL concentration. The mean absorbance values recorded for various test groups were compared using one-way analysis of variance (ANOVA) (Fig. 1). On comparison

Table 1: The mean absorbance values and percentage of cell viability of human primary pulp fibroblasts in individual groups as measured by MTT assay at 24 hours

Groups	*N*	*Mean absorbance values (Mean ± standard deviation)*	*Percentage cell viability*
Untreated control	3	0.84400 ± 0.00916	100
Toxic control	3	0.26433 ± 0.00650	31.27
1000 µg/mL GE	3	0.82233 ± 0.00907	97.43
500 µg/mL GE	3	0.82700 ± 0.00900	97.98
250 µg/mL GE	3	0.83033 ± 0.00950	98.38
125 µg/mL GE	3	0.83367 ± 0.00802	98.77
62.5 µg/mL GE	3	0.83933 ± 0.00550	99.44

GE, Garlic extract

Table 2: The mean absorbance values and percentage of cell viability of human primary pulp fibroblasts in individual groups as measured by MTT assay at 48 hours

Groups	*N*	*Mean absorbance values (Mean ± standard deviation)*	*Percentage cell viability*
Untreated control	3	1.22767 ± 0.01106	100
Toxic control	3	0.10300 ± 0.00953	8.38
1000 µg/mL GE	3	1.14367 ± 0.00416	93.15
500 µg/mL GE	3	1.17867 ± 0.00611	96.00
250 µg/mL GE	3	1.19600 ± 0.00200	97.17
125 µg/mL GE	3	1.22167 ± 0.00971	99.51
62.5 µg/mL GE	3	1.22567 ± 0.01006	99.83

GE, Garlic extract

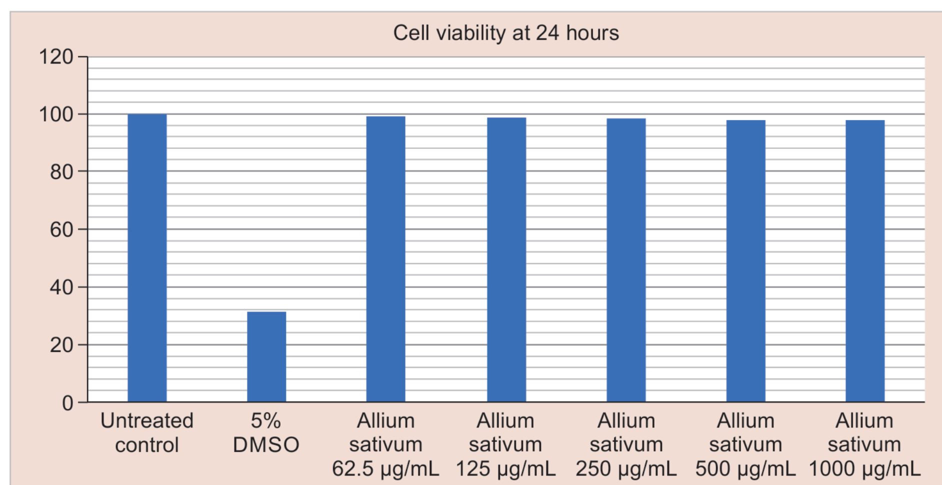

Fig. 1: Percentage of cell viability of human primary dental pulp fibroblasts in individual groups as measured by MTT assay at 24 hours

with the untreated control, no statistically significant difference in mean absorbance values was observed for all the concentrations of *A. sativum* tested, indicating biocompatibility of garlic extract against primary pulp fibroblasts. The mean absorbance values and percentage of cell viability reduced with increasing concentrations of *A. sativum* extract. However, this reduction was not statistically significant at 24 hours.

Cytotoxicity of Various Concentrations of *A. sativum* Extract on Primary Pulp Fibroblasts at 48 Hours

The mean absorbance value of untreated control wells was recorded as 1.22767 ± 0.01106 with 100% cell viability at 48 hours. Among all the concentrations of garlic extract tested, highest mean absorbance value of 1.22567 ± 0.01006 with 99.83% cell viability was recorded for 62.5 µg/mL concentration, while lower mean absorbance value of 1.14367 ± 0.00416 with 93.15% cell viability was recorded for 1000 µg/mL concentration. The mean absorbance values recorded for various test groups were compared using one-way ANOVA (Fig. 2). On comparison with untreated control group, all the concentrations of garlic extract except that of 125 µg/mL and 62.5 µg/mL showed a significant decrease in mean absorbance values. However, the percentage of cell viability was maintained high with all the tested concentrations.

Discussion

The present study was conducted to determine the cytotoxicity of *A. sativum* extract when used as a pulpotomy medicament for capping the remaining radicular portion of the pulp in primary teeth. An ideal pulp capping material should exhibit good biological and physical properties, protecting the tissues from microbial infections, reducing inflammation and being biocompatible, thereby aiding in healing of the remaining vital tissues of the primary tooth.

A. sativum generally known as "garlic" has been extensively researched in the medical literature. It is known to possess antibacterial,[11] antifungal,[26] antioxidant,[13] wound healing,[27,28] anticancer,[29] analgesic and anti-inflammatory,[30] antidiabetic, antihypertensive, immunomodulatory and neuroprotective properties.[31] The antimicrobial activity of garlic extract against oral microflora has been well established and several reports can be found in the literature corroborating the same.[20,32–34] Along with its use as a root canal irrigant,[16] intracanal medicament,[19] and mouthwash,[20] *A. sativum* has also been used as a medicament in vital pulp therapy of primary teeth.

Mohammad et al.[21] reported a success rate of 90% with *A. sativum* and 82% with formocresol in vital primary teeth pulpotomy. Kahvand et al.[22] found a clinical success rate of 100% in both *A. sativum* and formocresol group. Mohammad et al.[35] reported a success rate of 95% with *A. sativum* oil and 80% with formocresol.

Schmalz[36] in his review on biocompatibility testing of dental restorative materials, outlined the international standards for testing the biological properties of medical devices (e.g., dental materials) (ISO 7405) which states that any dental material should be subjected to three steps in a sequence, from initial *in vitro* tests (cytotoxicity, mutagenicity) to animal tests and from preclinical to clinical testing in humans. Therefore, it becomes imperative to test the biocompatibility of garlic extract on dental pulp tissue prior to its clinical usage as a vital pulp therapy medicament.

The *in vitro* biocompatibility tests are designed to simulate biological reactions to materials when placed on or into the tissues of the body. The assessment of biocompatibility in terms of cytotoxicity provides a measure of cell death when exposed to the material. Therefore, cytotoxicity testing using cell lines derived from human dental pulp tissue is appropriate while testing the materials used for vital pulp therapy procedures.[25]

In the present study, the cytotoxicity was determined using MTT assay. MTT assay is a colorimetric assay which is based on the conversion of tetrazolium salt into formazan crystals by mitochondrial dehydrogenase enzyme present in living cells. The dead cells lose the ability to reduce the tetrazolium salts into colored formazan products, while viable cells with active metabolism converts MTT into purple color formazan products with an absorbance at 570 nm. Thus, greater the intensity of the color produced, higher are the number of viable cells in the culture.[24]

In the present study, the MTT assay results revealed that *A. sativum* extract, in all the concentrations tested at both time intervals maintained a cell viability of greater than 90%. According to Tabatabaei et al.,[37] the percentage of cell viability over 90% indicates nontoxicity, between 60 and 90% indicates mild cytotoxicity, 30 and 60% indicates moderate toxicity while the

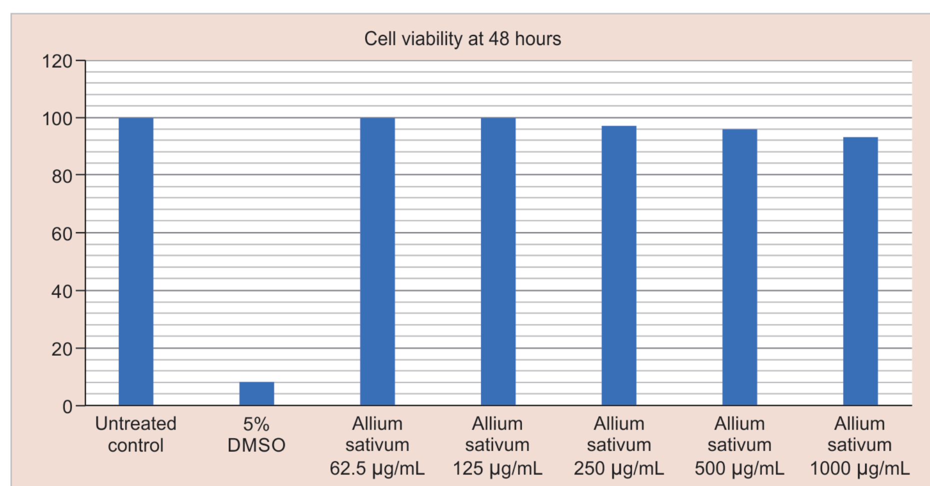

Fig. 2: Percentage of cell viability of human primary dental pulp fibroblasts in individual groups as measured by MTT assay at 48 hours

cell viability of <30% indicates severe toxicity. Therefore, from the present study results, it can be inferred that *A. sativum* extract is noncytotoxic on human primary dental pulp fibroblasts.

The cell viability did not seem to be affected by the concentration of the *A. sativum* extract at 24 hours. However, at 48 hours, the sensitivity of the cells seems to be dependent on the concentration of *A. sativum* with a decrease in the viability of cells noted with the increase in concentration.

The present study results indicating the noncytotoxic nature of garlic extract on human dental pulp fibroblasts can be supported with the findings of Mohammad et al.[38]

Bramanti et al.[39] in their study reported that garlic extract increased viability and stimulated cell growth leading to proliferation of human gingival fibroblast cells when subjected to MTT assay. Ozan et al.[40] reported mild cytotoxicity of garlic extract at a concentration of 12.5% on human gingival fibroblasts while the other tested concentrations of 6.25, 3.12, 1.5, and 0.6% were noncytotoxic. However, the cytotoxicity observed with 12.5% garlic extract was less when compared to chlorhexidine gluconate. In the present study, all the tested concentrations of 100, 50, 25, 12.5, and 6.25% of garlic extract did not exhibit any cytotoxicity at both 24 and 48 hours on human primary pulp fibrobalsts.

The favorable response of cells to garlic extract observed in the present study could be due to the synergistic actions of different compounds present naturally in garlic contributing to its antioxidant, anti-inflammatory, antimicrobial, and wound-healing properties. Garlic contains numerous potentially active constituents such as alliin, allicin, ajoene, diallyl trisulfide, s-allylcysteine, enzymes such as alliinase, myrosinase, peroxidases, and amino acids like arginine with few of trace minerals like selenium.[31] In addition to these, garlic also contains phenolic compounds and nonsulfur compounds like saponins.

The antioxidant property of garlic has been widely documented. The components derived from garlic bulbs and various extracts of garlic such as allicin, organosulfur compounds like allylcysteine, flavonoids, selenium are proven to prevent oxidative modification of deoxyribonucleic acid (DNA), proteins and lipids by scavenging reactive oxygen species (ROS), enhancing the expression of cellular antioxidant enzymes and increasing the glutathione levels in the normal cells.[32] Szychowski et al.[29] reported that garlic extract protects cells from ROS damage by not just capturing ROS directly, but also by stimulating antioxidant gene expression thereby activating the cellular antioxidant system. Shrivastava et al.[41] has proven garlic to exhibit tumor inhibitory and normal cell protection properties.

The antimicrobial activity of garlic against various oral microflora has been extensively documented[12,20,33,34] and is attributed to the presence of sulfides. Tsao and Yin[11] reported that the active component allicin and various diallyl sulfides present in garlic are responsible for its strong antimicrobial and antifungal activity.

In addition, garlic is known to exhibit potent analgesic and anti-inflammatory properties. Ajoene and diallyl disulfide components of garlic are known to exhibit strong inhibitory action on prostaglandin synthesis and 5-lipoxygenase.[30] Allicin and ajoene also inhibit nitric oxide, a pro-inflammatory mediator produced by macrophages.

Considering the findings of the present study proving its biocompatibility, the ease of availability, cost-effectiveness, and biological activities, *A. sativum* can be considered a vital pulp therapy medicament in primary teeth.

Limitations of the Study

It is important to consider that the assessment was based on *in vitro* experimental technique conducted in a controlled environment and certain caution needs to be exerted while extrapolating the same to clinical conditions.

Though MTT assay is proven to be a sensitive and effective method in determining the cell viability, it is preferable to apply more than one assay to obtain reliable results.

Conclusion

A. sativum extract is noncytotoxic in nature and preserves the vitality of cultured human primary dental pulp fibroblasts making it a suitable material for use in vital pulp therapy procedures of primary teeth.

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