Original Article

"Comparative evaluation of cytotoxicity of three herbal endodontic irrigants at three intervals of time" – An *in vitro* study

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Abstract

Aim: The aim of the study was to evaluate and compare the cytotoxicity of 25% of neem leaf extract (*Azadirachta indica*), 20% of guava leaf extracts (*Psidium guajava*), and 20% of cinnamon extract (*Cinnamomum zeylanicum*) irrigants at three intervals of time.

Methodology: Four groups were formed ($n = 15$), Group 1 (control group) – normal saline solution, Group 2 – 25% of neem extract, Group 3 – 20% of guava extract, and Group 4 – 20% of cinnamon extract. Each group was further divided into three subgroups based on intervals ($n = 5$). Subgroup A – at 10 min, Subgroup B – at 20 min, and Subgroup C – at 30 min. One hundred microliters of each irrigant was added to 2 mL of the diluted red blood cells suspension obtained from a human volunteer. Hemoglobin (Hb) estimation was done with an automated hematology analyzer after incubating the test samples at 10, 20, and 30 min intervals.

Results: The reduction in the mean Hb values was not statistically significant in the normal saline, guava, and cinnamon groups. However, in the neem extract group, the mean Hb values reduced significantly at $P < 0.001$. Among the subgroups, Subgroup A (10 min) showed the least cytotoxicity.

Conclusion: In the present study, 20% guava extract had the lowest cytotoxicity and cytotoxicity increased with time. Hence, 20% guava extract can be used as an alternative to conventional irrigants as it has been shown to have the least cytotoxicity.

Keywords: *Azadirachta indica*; *Cinnamomum zeylanicum*; cytotoxicity; herbal endodontic irrigants; *Psidium guajava*

INTRODUCTION

For successful endodontic therapy, disinfection is crucial. Effective eradication of microbes requires thorough cleaning and shaping of the root canals, along with proper disinfection protocols.[1] Irrigation dynamics plays a significant role in this process.[2,3] Synthetic antimicrobial irrigants have been widely used in endodontics, but concerns about antibiotic resistance and harmful side effects are driving interest in herbal antibacterial alternatives. These alternatives are valued for their perceived affordability, safety, and effectiveness in root canal therapy.[3]

An ideal endodontic irrigant must be nontoxic to vital tissues and noncaustic to periodontal tissues, especially in cases of blunderbuss canals, perforations, and improper techniques that can lead to the permeation of solution into surrounding tissues.[4,5] Various methods have been employed to evaluate the cytotoxicity of endodontic irrigants. Pashley *et al*. evaluated the cytotoxicity on red

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blood corpuscles, Faria *et al*. and Zhang *et al*. evaluated the cytotoxicity on L929 fibroblasts and Barnhart *et al*. on gingival fibroblasts.[4]

Neem is known for its broad range of antiviral, antifungal, and antibacterial properties. Hence, neem is also considered a potent agent for use in root canal irrigation.[6]

Psidium guajava, commonly known as guava, is a phytotherapeutic plant with a wide range of medicinal properties. One of the significant attributes of guava is its antioxidant properties, which are crucial in preventing and managing various human diseases by scavenging reactive oxygen species.[7,8]

Cinnamon, also known as *Cinnamomum zeylanicum,* has gained popularity recently due to its antibacterial activity. This action is attributed to the presence of vanillic, caffeic, gallic, protocatechuic, *p*-coumaric, and ferulic acids found in its extracts.[9-13]

In addition to antibacterial efficacy, an endodontic irrigant should be nontoxic to periodontal tissue and periapical cells. Few studies have investigated the cytotoxicity of herbal irrigants.[4,5] Studies indicate that irrigant's cytotoxicity can fluctuate over time. Therefore, it is crucial to assess the cytotoxicity of endodontic irrigants at various time intervals to ensure their safety and effectiveness.[5,14] The minimum inhibitory concentration for neem, cinnamon, and guava was found to be 25%, 20%, and 20%,[6,15] hence, the same was used in the present study.

Hence, the study aimed to evaluate and compare the cytotoxicity of 25% neem leaf extract (*A. indica*), 20% guava leaf extract (*P. guajava*), and 20% cinnamon extract (*C. zeylanicum*) irrigants at three intervals of time.

METHODOLOGY

Sixty test tubes containing diluted red blood cell (RBC) suspension were prepared.

Preparation of 25% ethanolic neem and 20% ethanolic cinnamon extracts

The commercially available neem and cinnamon powder was mixed with 50% ethanol and stirred on a magnetic stirrer for 4 h. The mixture was then filtered using Whitman's filter paper to obtain the filtrate, which was subsequently dried to yield the extract. For the preparation of the irrigating solution, 25 g of crude neem extract and 20 g of crude cinnamon extract were separately dissolved in 100 mL of dimethyl sulfoxide solution each.[15]

Preparation of 20% ethanolic guava leaf extract

The commercially available organic guava leaf powder was mixed with ethanol and allowed to stand for 48 h at room temperature. Subsequently, the mixture underwent Soxhlet extraction, operating at four cycles per hour for a total of 12 h. For preparing the irrigating solution, 20 g of the crude guava leaf extract was mixed with 100 ml of dimethyl sulfoxide solution.[16] This method minimizes solvent usage, enhances efficiency in extracting less soluble compounds, and ensures thorough extraction compared to standard soaking methods.

Division of study groups

Four groups were formed (n = 15): Group 1 (control group) – normal saline solution, Group 2 – 25% neem extract, Group 3 – 20% guava extract, and Group 4 – 20% cinnamon extract [Figure 1].

Each group was further divided into three subgroups containing five samples each based on three intervals of time at which cytotoxicity evaluation was done. Subgroup A – at 10 min, Subgroup B – at 20 min, and Subgroup C – at 30 min.

% Hemoglobin estimation

RBCs were chosen to evaluate cytotoxicity. Fresh blood from the human volunteer was drawn into ethylenediaminetetraacetic acidcontainers and spun at 1000 rpm for 10 min, plasma was discarded and the packed cell volume obtained was washed twice in Dulbecco's Phosphate-Buffered Saline by centrifugation. The final hematocrit of RBC suspension was adjusted to 45%. One hundred microliters of each irrigant was added to 2 mL of the diluted RBC suspension in individual test tubes separately.

Hemoglobin (Hb) estimation was done with an automated hematology analyzer after incubating the test samples for 10 min, 20 min, and 30 min [Figure 2].

Subgroup A – after incubating for 10 min, tubes were centrifuged at 1000 rpm for 10 min. Subgroup B – after incubating for 20 min, tubes were centrifuged at 1000 rpm for 10 min. Subgroup C – after incubating for 30 min, tubes were centrifuged at 1000 rpm for 10 min.

The supernate volume obtained was subjected to Hb estimation measured by an automated hematology analyzer in all subgroups.

Statistical analysis

The Statistical Package for the Social Sciences for Windows Version 22.0 Released 2013. Armonk, NY: IBM Corp., was used to perform statistical analysis. One-way analysis of variance (ANOVA) test followed by Tukey's *post hoc* test was used to compare the mean Hb values between four groups at different time intervals. Repeated measures of ANOVA followed by Bonferroni's *post hoc* test were used

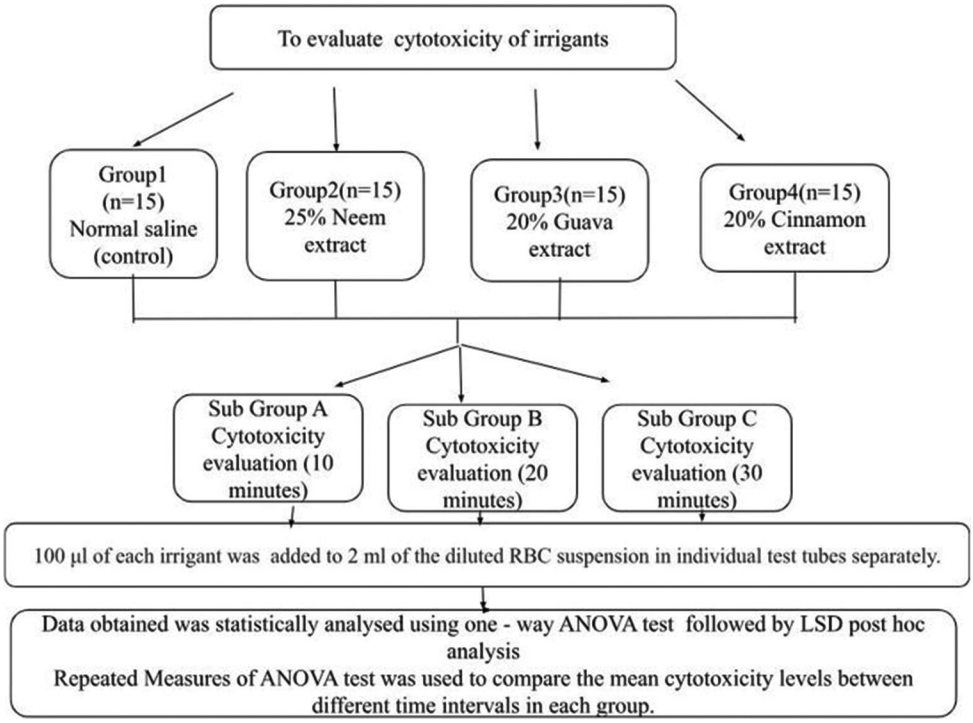


Figure 1: Division of experimental groups. RBC: Red blood cell, ANOVA: Analysis of variance, LSD: Least significant difference

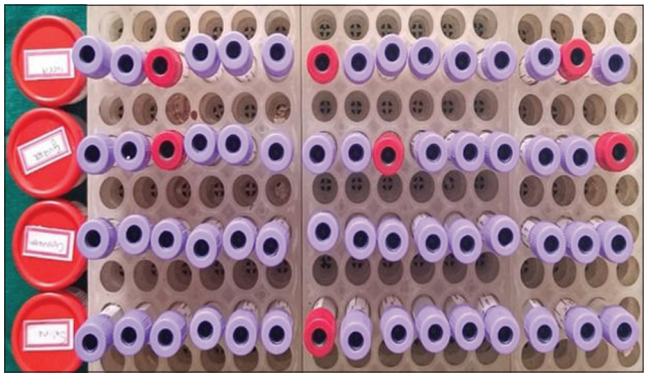


Figure 2: Test samples incubated for 10 min, 20 min, and 30 min

to compare the mean Hb values between different time intervals in each group. The level of significance was set at $P < 0.05$.

RESULTS

The mean Hb value after 10 min (Subgroup A) in all four groups was statistically significant at $P = 0.04$ [Table 1 and Graph 1].

Multiple comparisons of the mean difference between the control group and experimental groups in Subgroup A revealed that the neem group showed significantly lesser mean Hb at $P = 0.04$. However, no significant difference in the mean Hb values was found between other groups.

The mean Hb value after 20 min (Subgroup B) was statistically significant at $P = 0.007$ [Table 1 and Graph 1]. Multiple comparisons of the mean difference between control and experimental groups revealed that the neem group showed significantly lesser mean Hb values at $P = 0.01$. However, no significant difference in the mean Hb values was found between other groups.

The mean Hb value after 30 min (Subgroup C) in all four groups was statistically significant at $P = 0.004$ [Table 1 and Graph 1]. Multiple comparisons of the mean difference between the control group and experimental groups revealed that the neem group showed significantly lesser mean Hb at $P = 0.006$. However, no significant difference in the mean Hb values was found between other groups.

DISCUSSION

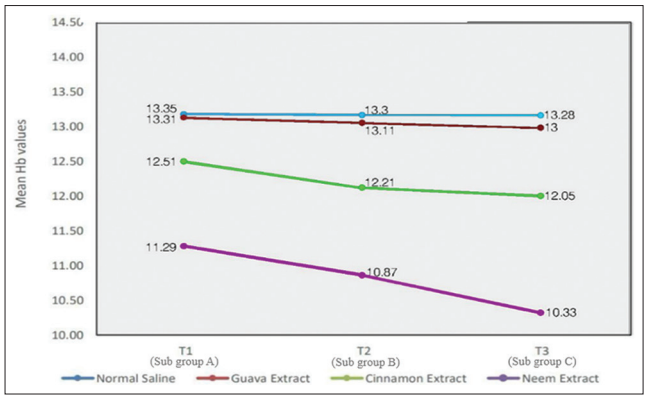
The ideal endodontic irrigating solution should effectively act as an antimicrobial agent while exhibiting low toxicity to periapical tissues.[17] Commercially available irrigants that are used in endodontic therapy can potentially cause damage or irritation, leading to degeneration of periapical tissue and delayed wound healing.[4] Herbal extracts have antimicrobial action and exhibit low toxicity to periapical tissues also remove the smear layer formed on the dentin surface after instrumentation.[18]

RBC was selected as a biological model to assess cytotoxic effects[4] in the present study because they can be easily isolated using minimally invasive procedures. Their

Table 1: Comparison of mean Hb values after 10, 20 & 30 mins between 4 groups using one way ANOVA test

Groups	*n*	Mean	SD	Minimum	Maximum	*P*
Comparison of mean Hb values after 10 min (Subgroup A) between 4 groups using one-way ANOVA test						
Normal saline (Group 1 control)	15	13.35	2.60	9.1	18.3	0.04*
Guava extract (Group 3)	15	13.31	2.91	9.1	18.3	
Cinnamon extract (Group 4)	15	12.51	1.86	8.8	15.7	
Neem extract (Group 2)	15	11.29	1.07	9.5	13.2	
Comparison of mean Hb values after 20 min (Subgroup B) between 4 groups using one-way ANOVA test						
Normal saline (Group 1 control)	15	13.33	2.22	8.8	17.9	0.007*
Guava extract (Group 3)	15	13.11	2.63	8.8	17.9	
Cinnamon extract (Group 4)	15	12.21	1.62	8.5	15.2	
Neem extract (Group 2)	15	10.87	1.02	8.1	12.8	
Comparison of mean Hb values after 30 min (Subgroup C) between 4 groups using one-way ANOVA test						
Normal saline (Group 1 control)	15	13.28	2.1	7.9	17.1	0.004*
Guava extract (Group 3)	15	13	2.44	7.9	17.1	
Cinnamon extract (Group 4)	15	12.05	1.32	7.2	14.6	
Neem extract (Group 2)	15	10.33	0.9	6.5	11.9	

*Statistically Significant. Hb: Hemoglobin, SD: Standard deviation



Graph 1: Mean hemoglobin values between different time intervals in each group. Hb: Hemoglobin

semipermeable membranes establish an osmotic gradient that regulates fluid movement in and out of the cells. Hypertonic solutions cause rapid water efflux, leading to cell shrinkage (crenation) and collapse. Conversely, hypotonic solutions cause cells to swell and eventually burst (lyse), releasing their contents into the surrounding medium.[19] Using human RBC for cytotoxicity evaluation in the present study is justified because the intracellular Hb content can be quantitatively measured.[4] A lower percentage of Hb indicates higher cytotoxicity of the irrigating solution, making it a reliable indicator of the solution's impact on cellular integrity and function.

In this study, the cytotoxicity of the irrigants among the experimental groups was evaluated. The 20% guava extract group exhibited the least cytotoxicity, followed by the 20% cinnamon extract and 25% neem extract groups. The normal saline (control group) showed the least cytotoxicity at 10, 20, and 30 min intervals. Cytotoxicity was lowest at 10 min (Subgroup A) and increased progressively with longer durations, peaking at 30 min (Subgroup C).

In the neem extract group, %Hb values significantly decreased with an increase in time and the difference was statistically significant. This was in accordance with a study done by Allayl *et al*. which showed the highest cytotoxicity with 25% aqueous neem leaf extract at all tested periods.[5] Similar results were seen in a study conducted by Arévalo-Híjar *et al*., in which *A. indica* showed higher cytotoxicity than *Moringa oleifera*.[20] The highest cytotoxicity shown by the neem extract could be due to increased reactive oxygen species production and mitochondrial fragmentation. It results in the decrease of oxidative phosphorylation complex 1 (mitochondrial nicotinamide adenine dinucleotide hydrogen (NADH)-ubiquinone oxidoreductase) and the loss of Molecular Weight of Fibrinogen-Elevated (MWFE) protein, which in turn activates caspases.[21]

The guava extract group showed significantly lesser cytotoxicity in comparison to neem extract. This was in accordance with a study done by Senthilkumar and Ramesh where guava leaf extract showed mild cytotoxicity in comparison to sodium hypochlorite (NaOCl) on the L929 fibroblast cell line. Another conclusion from the same study was that the cytotoxicity of guava extract was based on its concentration ranging from no to mild cytotoxic effect.[22] In the present study, cinnamon extract showed slightly more cytotoxicity in comparison to guava extract and the control group (normal saline) which was not statistically significant. This was in accordance with LeBel *et al*. who concluded that cinnamon oil has no toxic effects on oral keratinocytes.[23]

In this study, mean Hb values decreased over time in each group, indicating cytotoxicity possibility due to hemolysis and protein loss from RBC membranes caused by the solution's oxidizing effects on the cell membrane of RBCs. The increase in cytotoxicity with an increase in time was not statistically significant, this may be due to the smaller time intervals taken in the present study, the concentration

taken, and the composition of the irrigants used in the present study. A study conducted by Salem *et al*., where they evaluated the cytotoxicity of irrigants at 1-, 5-, and 15-min time intervals revealed that bleached turmeric extract had significantly higher cell viability percent than NaOCl at all-time intervals, but cell viability percent significantly decreased over time.[24] This indicates that although herbal irrigants are less cytotoxic than conventional irrigants, their cytotoxicity increases with increase in time. Another study by Karkehabadi *et al*. also concluded that the mean percentage of viable cells significantly decreased over time,[25] indicating that cytotoxicity increases with time. This increase in cytotoxicity with time exhibited by the neem extract group could be attributed to the synergistic effect of ethanol and the cytotoxic effect of neem. Studies indicate that ethanol concentrations above 25%–30% can cause alterations in cellular basement membranes, affecting cell viability in a dose- and time-dependent manner.[22]

Limitations and future scope

This *in vitro* study, which had a limited sample size, encountered a major challenge with herbal extracts due to their need for fresh preparation and potential modifications to improve taste and acceptability. The current research focused on assessing cytotoxicity in RBCs as an initial trial. To better determine the potential of guava and cinnamon for use in root canal disinfection, additional studies are necessary to evaluate their biocompatibility and efficacy in this context.

CONCLUSION

Within the limitations of the study following conclusions were drawn.

In the experimental group, 20% guava extract showed the least cytotoxicity followed by 20% cinnamon extract and 25% neem extract. Moreover, cytotoxicity is time-dependent and it increases with increased time. Hence, 20% guava extract can be used as an alternative to conventional irrigants.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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