Original Article

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Dentistry Section

Histological Evaluation of the Effectiveness of Four Decalcifying Solutions on Teeth and Bone in Rats: An In-vitro Study

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ABSTRACT

Introduction: Intense histological research has been conducted on human bone and teeth for a long period of time. Histological evaluation of these highly mineralised tissues demands a thorough decalcification process using different chemical agents, which might alter some aspects of tissue architecture and staining properties.

Aim: To assess the impact of four decalcifying agents on rat teeth and bone, focusing on the rate of decalcification, staining effectiveness, and tissue structure preservation.

Materials and Methods: An in-vitro animal study was conducted in the Department of Oral Diagnosis at the College of Dentistry, University of Baghdad, Bab Almoaadim, Baghdad, Iraq from October 2022 to March 2023. Four decalcification agents, namely 10% Neutral Ethylenediaminetetraacetic Acid (EDTA), 10% formic acid, 3% nitric acid, and 5% nitric acid, were used to decalcify 24 molar teeth with their surrounding alveolar bone obtained from healthy male rats, which were randomly divided into four groups, each group consisting of six teeth. The decalcified sections underwent regular processing, and staining was performed using Haematoxylin and Eosin (H&E). Grading was conducted after two separate observers examined the stained sections under a light microscope. Data were presented using Mean±Standard Deviation (SD), number, and percentage. A one-way Analysis of Variance (ANOVA) (F-test) was used to compare the decalcification times within groups. Several group comparisons, including categorical data, were examined using the Chi-square test.

Results: Nitric acids (3% and 5%) exhibited the fastest decalcification with a mean value of (2±0.89) days, while Formic acid required (3.5±0.54) days, and EDTA was the slowest, with (19±1.09) days. These differences in decalcification time were found to be statistically significant ($p<0.00001$). Formic acid 10% demonstrated superior tissue preservation and staining quality, with excellent staining results, minimal dentin-pulp separation, and preservation of pulp zones, cementum, and osteoblasts. In contrast, nitric acid 5% resulted in severe dentin-pulp separation, absence of pulp zones, absence of osteoblast, and significant osteocyte retraction. Statistical significance was observed across all agents for dentin-pulp separation, pulp organisation, cementum destruction, Periodontal Ligament (PDL) separation, and osteocyte retraction (p-values of 0.004, 0.02, 0.02, 0.04, and 0.03, respectively).

Conclusion: Consequently, 10% formic acid emerged as the most efficient decalcifying solution, ensuring rapid decalcification with favourable staining intensity and tissue architecture.

Keywords: Formic acid, Neutral Ethylenediaminetetraacetic acid, Nitric acid, Tissue architecture

INTRODUCTION

Histological analysis of mineralised tissues such as bone and teeth requires efficient decalcification to enable microscopic examination. Alveolar bone, dentin, cementum, and enamel are among the hard mineralised components of teeth, while dental pulp and PDL are among the soft organic components [1]. Decalcification, as an initial histological step, involves the removal of calcium ions or salts to make the tissue amenable to microscopic assessment [2].

A variety of decalcification solutions exist, ranging from strong acids like nitric acid [3] and hydrochloric acid [4], weak acids such as formic acid [5], and Tricarboxylic acid [6], chelating agents like EDTA [7], or a combination of solutions [8,9]. However, these solutions can compromise tissue integrity, requiring a delicate balance between effective decalcification and tissue preservation. To process paraffinised tissue samples effectively, an efficient decalcifying technique must be developed. Preserving tissue architecture with a short process time represents an optimal decalcifying agent [10,11].

Various decalcification agents have been studied in previous literature. Sangeetha R et al., reported that both formic acid and EDTA exhibit good tissue preservation and staining effectiveness [12]. Conversely, Zappa J et al., found that formic acid and nitric acid showed the worst decalcification results for both the hard and soft-tissue components of teeth when compared to EDTA and other agents [13]. Additionally, Sanjai K et al., reported that EDTA produced the best overall results [14].

The present study focuses on evaluating the effectiveness of four decalcifying solutions on teeth and bone in rats through histological evaluation. While previous studies may have focused on various decalcification methods, the present research specifically examines the impact of these unique decalcifying solutions at specific concentrations on the histological features of teeth and the surrounding alveolar bone in rats. Notably, there is a lack of prior research evaluating these particular agents in this context. The findings of the present study could provide valuable insights into the optimal decalcification agents that yield complete decalcification in the shortest time with the best stained sections possible while preserving the hard and soft-tissue structure for future research involving rat teeth and bone, potentially enhancing the accuracy and reliability of histological analysis in this context.

MATERIALS AND METHODS

An in-vitro animal study was conducted in the Department of Oral Diagnosis at the College of Dentistry, University of Baghdad, Bab Almoaadim, Baghdad, Iraq from October 2022 to March 2023. Institutional Ethics Committee (IEC) approval (IEC No. 671, Date: 13/10/2022) was obtained before conducting the study.

A total of 24 molar teeth with their surrounding alveolar bone, obtained from healthy male albino rats (16 weeks old, weighing 300±10 g), were used to collect fresh tooth and mandibular tissue samples. These samples were randomly divided into four groups, with each group consisting of six teeth. The rats were housed in polycarbonate cages on a bed of wood shavings in an animal facility. They were fed rat chow pellet food and had unrestricted access to tap water. The animals were kept under regular laboratory conditions with a temperature of 25±2°C and a 12-hour light/dark cycle.

Inclusion criteria: The study exclusively enrolled healthy male albino rats that were 16 weeks of age and had intact molar teeth and alveolar bone.

Exclusion criteria: The study specifically excluded unhealthy female or male rats under the age of 16 weeks, as well as any teeth or alveolar bone that were damaged during tissue collection.

Study Procedure

A total of 24 molar teeth, along with their supporting tissues (cementum, PDL, and alveolar bone), were collected and subjected to different decalcification solutions (10% EDTA, 3% nitric acid, 5% nitric acid, and 10% formic acid). The tissues were fixed in 10% formalin for 24 hours, followed by the decalcification process. Decalcification was carried out at room temperature by placing the teeth in a container with a thread and immersing them in approximately 100 mL of solution. The start time of decalcification was recorded. All solutions were changed every 48 hours until complete decalcification was achieved. The time required for decalcification was determined by physically probing the tissue with a needle [15]. The decalcified specimens underwent standard tissue processing, were embedded in paraffin wax, sectioned, and stained with H&E for microscopic evaluation. The study's assessment criteria included the evaluation of decalcification time, staining intensity, and histological details of both soft and hard tissues. Each specimen in the decalcifying solutions was shaken daily, aiding in the effective decalcification of the samples and preservation of their tissue structure [16].

Histological examination: A light microscope was utilised to observe the stained sections. The effectiveness of the various decalcifying agents used in the study was assessed and graded according to the following criteria [17]:

1. The staining intensity was evaluated and categorised as adequate, under-stained, or over-stained.
2. The effects on the histological details of tissues, such as fixation, processing, cutting method, and staining duration, were considered, and standardised approaches were followed to maintain consistency.

Regarding the specific tissues evaluated:

- The interface between pulp and dentin was examined under a light microscope, and their separation was graded as absent, mild, moderate, or severe depending on the degree of separation.
- Dental pulp was scrutinised for the presence or absence of all four pulp zones and any separation from the surrounding dentin.
- Dentin was inspected for the presence of vapour bubbles and fraying in the dentinal tubules, indicating any potential negative impact from the decalcifying solutions, and described as absent or present.
- The architecture of the cementum was assessed for any loss or destruction and described as absent or present.
- The attachment of the PDL to the surrounding bone and teeth was examined for detachment and categorised as absent, mild, moderate, or severe.
- The condition of the alveolar bone was evaluated by observing the presence or absence of osteoblasts lining the trabeculae and the retraction of osteocytes within the lacunae, categorised as <50% of cells retracting and ≥50% of cells retracting.

STATISTICAL ANALYSIS

Results were analysed using Statistical Package for Social Sciences (SPSS) software version 25.0 The data were presented as Mean±SD, number, and percentage. A one-way Analysis of Variance (ANOVA) (F-test) was employed to compare decalcification times between groups and identify any differences. The Chi-square test was utilised to analyse multiple group comparisons involving categorical data. For statistical significance, a p-value of 0.05 or less was considered.

RESULTS

1. **Time for decalcification:** The study results revealed that the shortest duration for complete decalcification was observed in both nitric acid concentrations (3% and 5%), with a mean time of (2±0.89) days. In contrast, decalcification using 10% EDTA took the longest time, (19±1.09) days, while decalcification with 10% formic acid required (3.5±0.54) days. These differences in decalcification time were found to be statistically significant (p<0.00001), as shown in [Table/Fig-1].

Decalcification agents	Mean±SD
EDTA 10%	19±1.09
Formic acid 10%	3.5±0.54
Nitric acid 3%	2±0.89
Nitric acid 5%	2±0.89

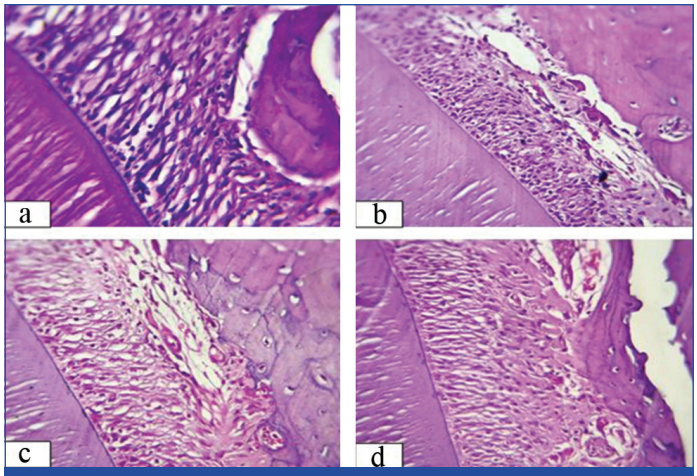
[Table/Fig-1]: Duration of the decalcification process in days.
One-way ANOVA (F=530.81) was used and results were expressed as Mean±SD, the p-value is <0.00001 and the result is significant at p<0.05. SD: Standard deviation

2. **Staining intensity:** In [Table/Fig-2,3a-d], specimens decalcified with 10% formic acid showed the highest percentage (N=5, 83.3%) of adequate staining with H&E, followed by nitric acid at both 3% and 5% (n=4, 66.7%) for each group, and then EDTA at 10% (n=1, 16.16%). EDTA at 10% exhibited the highest percentage of overstaining (n=4, 66.7%). These findings were not significant among all the agents (p-value=0.30).

Decalcification agents	Staining intensity			
	Undertrained n (%)	Overstrained n (%)	Adequate n (%)	Total
EDTA 10%	1 (16.6)	4 (66.7)	1 (16.6)	6
Formic acid 10%	1 (16.6)	0	5 (83.3)	6
Nitric acid 3%	2 (33.3)	0	4 (66.7)	6
Nitric acid 5%	1 (16.6)	1 (16.6)	4 (66.7)	6

[Table/Fig-2]: Chi-square among groups for staining intensity.
The Chi-square statistic is 2.1721. The p-value is 0.30.
The result is not significant at p<.05

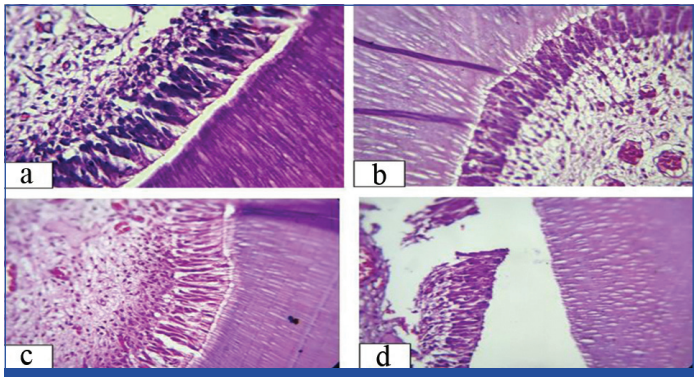
3. **Dentin-pulp separation:** Microscopic examination revealed that 4 (66.7%) specimens decalcified with 10% formic acid had no dentin-pulp separation, followed by nitric acid at 3% (n=3, 50%). Severe dentin-pulp separation was observed in 4 (66.7%) of specimens decalcified with nitric acid at 5%. These findings were significant among all the agents (p-value=0.004) [Table/Fig-4,5a-d].
4. **Pulp organisation:** As shown in [Table/Fig-6], the pulp zones were present in 4 (66.7%) of specimens decalcified with EDTA 10%, formic acid 10%, and nitric acid 3%, while only 1 (16.6%) of specimens decalcified with nitric acid at 5% had pulp zones. Pulp zones were absent in 5 (83.3%) of specimens decalcified with nitric acid at 5%. These findings were significant among all the agents (p-value=0.02) [Table/Fig-6].



[Table/Fig-3]: (a) EDTA 10% overstraining; (b) Formic acid 10% adequate staining; (c) Nitric acid 3% adequate staining; (d) Nitric acid 5% adequate staining H&E (X40).

Decalcification agents	Dentin-pulp separation				Total
	Absent n (%)	Mild n (%)	Moderate n (%)	Sever n (%)	
EDTA 10%	0	2 (33.3)	3 (50)	1 (16.6)	6
Formic acid 10%	4 (66.7)	0	2 (33.3)	0	6
Nitric acid 3%	3 (50)	3 (50)	0	0	6
Nitric acid 5%	0	2 (33.3)	0	4 (66.7)	6

[Table/Fig-4]: Chi-square analysis for pulp-dentin separation.
The Chi-square statistic is 24. The p-value is 0.004. The result is significant at p<0.05

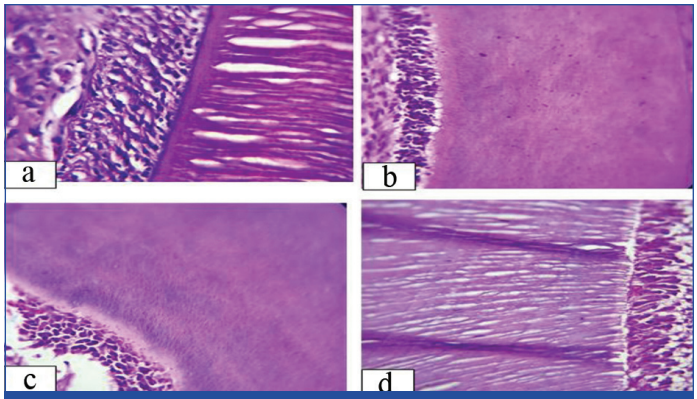


[Table/Fig-5]: (a) EDTA 10% moderate dentin–pulp separation with organised pulp tissue (presence of 4 pulp zones); (b) Formic acid 10% absent dentin–pulp separation with organised pulp tissue (presence of 4 pulp zones); (c) Nitric acid 3% absent dentin–pulp separation with organised pulp tissue (presence of 4 pulp zones); (d) Nitric acid 5% severe dentin-pulp separation with disorganised pulp tissue (absence of 4 pulp zones). H&E (X40).

Decalcification agents	Pulp organisation (presence of pulp zones)		Total
	Present n (%)	Absent n (%)	
EDTA 10%	4 (66.7)	2 (33.3)	6
Formic acid 10%	4 (66.7)	2 (33.3)	6
Nitric acid 3%	4 (66.7)	2 (33.3)	6
Nitric acid 5%	1 (16.6)	5 (83.3)	6

[Table/Fig-6]: Chi-square analysis for pulp zones organisation.
The Chi-square statistic is 14.5315. The p-value is 0.02. The result is significant at p<0.05

5. **Dentin destruction:** The microscopic examination of decalcified sections, as depicted in [Table/Fig-7a-d], revealed that the dentin was not destroyed in 4 (66.7%) of the specimens when treated with 10% formic acid and 3% nitric acid. In contrast, a higher percentage of dentin destruction 4 (66.7%) was observed in specimens decalcified with 5% nitric acid. These findings were not significant among all the agents (p-value=0.60) [Table/Fig-8].
6. **Cementum destruction:** Cementum was preserved in 5(83.3%) of specimens decalcified with 10% EDTA, 10% formic acid, and 3% nitric acid. Half of the specimens (3, 50%) decalcified with 5% nitric acid had disrupted cementum. These findings were significant among all the agents (p-value=0.02) [Table/Fig-9,10a-d].
7. **PDL separation:** The study found that 4 out of 6 specimens treated with 10% formic acid and 3% nitric acid did not show any separation of the PDL. In comparison, specimens treated with EDTA showed moderate PDL separation in 3 out of 6 cases. These findings were significant among all the agents (p-value is 0.04) [Table/Fig-10,11].
8. **Osteoblast surrounding bone trabeculae:** As shown in [Table/Fig-12,13], osteoblasts were present and



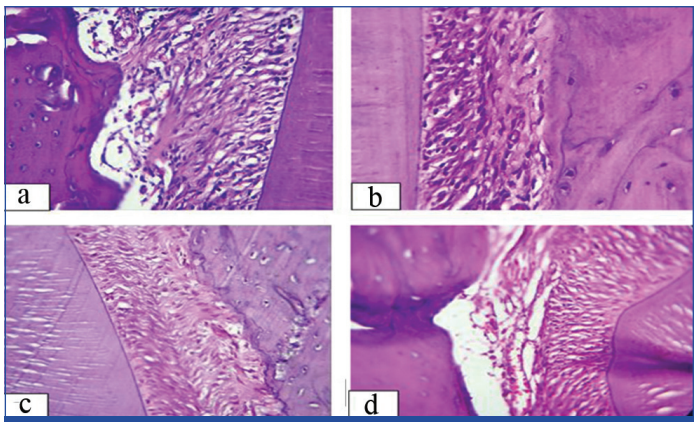
[Table/Fig-7]: (a) EDTA 10% present dentin destruction (frying in dentinal tubules); (b) Formic acid 10% absent dentin destruction; (c) Nitric acid 3% absent dentin destruction; (d) Nitric acid 5% present dentin destruction (frying in dentinal tubules) H&E (X40).

Decalcification agents	Dentin destruction		Total
	Present n (%)	Absent n (%)	
EDTA 10%	3 (50)	3 (50)	6
Formic acid 10%	2 (33.3)	4 (66.7)	6
Nitric acid 3%	2 (33.3)	4 (66.7)	6
Nitric acid 5%	4 (66.7)	2 (33.3)	6

[Table/Fig-8]: Chi-square for dentin destruction.
The Chi-square statistic is 1.8462. The p-value is .60.
The result is not significant at p<0.05.

Decalcification agents	Cementum destruction		Total
	Present n (%)	Absent n (%)	
EDTA 10%	1 (16.6)	5 (83.3)	6
Formic acid 10%	1 (16.6)	5 (83.3)	6
Nitric acid 3%	1 (16.6)	5 (83.3)	6
Nitric acid 5%	3 (50)	3 (50)	6

[Table/Fig-9]: Chi-square for cementum destruction.
The Chi-square statistic is 22.6667. The p-value is 0.02; The result is significant at p<0.05



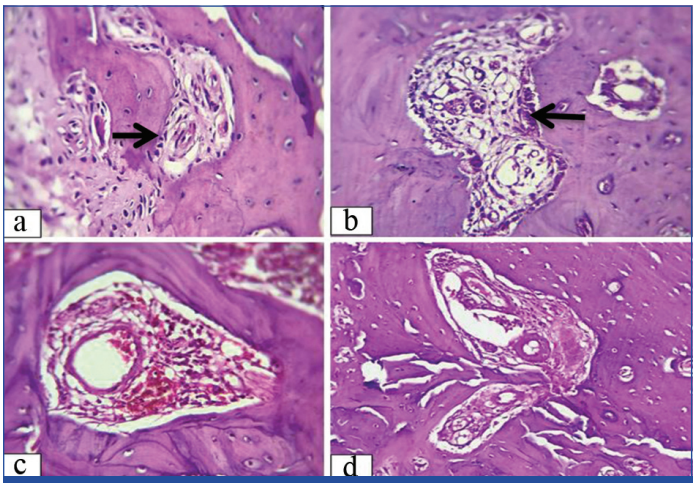
[Table/Fig-10]: (a) EDTA 10% absent cementum destruction with moderate PDL separation from bone; (b) Formic acid 10% absent cementum destruction with no PDL separation; (c) Nitric acid 3% absent cementum destruction with no PDL separation; (d) Nitric acid 5% absent cementum destruction with sever PDL separation from bone H&E (X40).

Decalcification agents	PDL separation from tooth structure or bone				Total
	Absent n (%)	Mild n (%)	Moderate n (%)	Sever n (%)	
EDTA 10%	2 (33.3)	0	3 (50)	1 (16.6)	6
Formic acid 10%	4 (66.7)	2 (33.3)	0	0	6
Nitric acid 3%	4 (66.7)	0	0	2 (33.3)	6
Nitric acid 5%	2 (33.3)	0	2 (33.3)	2 (33.3)	6

[Table/Fig-11]: Chi-square for PDL separation from tooth structure or bone. The Chi-square statistic is 14.0485. The p-value is 0.04. The result is significant at p<0.05

Decalcification agents	Osteoblast surrounding bone trabeculae		Total
	Present n (%)	Absent n (%)	
EDTA 10%	3 (50)	3 (50)	6
Formic acid 10%	4 (66.7)	2 (33.3)	6
Nitric acid 3%	2 (33.3)	4 (66.7)	6
Nitric acid 5%	2 (33.3)	4 (66.7)	6

[Table/Fig-12]: Chi-square for osteoblasts surrounding bone trabeculae. The Chi-square statistic is 1.8462. The p-value is 0.60. The result is not significant at p<0.05.



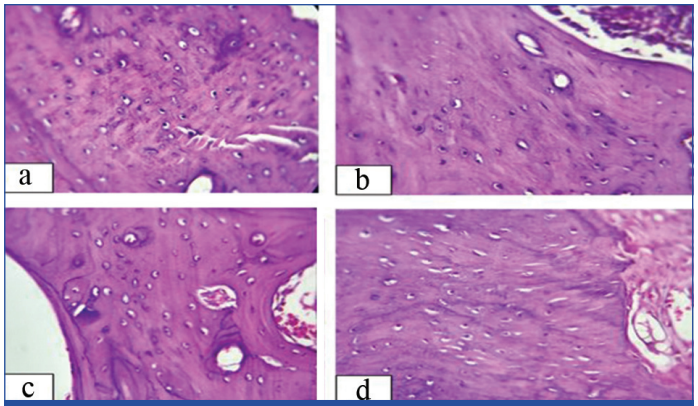
[Table/Fig-13]: (a) EDTA 10% present of osteoblast rimming; (b) Formic acid 10% present of osteoblast rimming; (c) Nitric acid 3% absent of osteoblast rimming; (d) Nitric acid 5% absent of osteoblast rimming. Arrow head (osteoblast surrounding bone trabecule) H&E (X40).

surrounded the bone trabeculae in four out of six specimens treated with 10% formic acid, but they were absent in four out of six specimens decalcified with 3% nitric acid and 5% nitric acid. Half of the specimens (three out of six) decalcified with EDTA preserved their osteoblasts. These findings were not significant among all the agents (p-value=0.60).

9. **Osteocyte retraction:** Retracted osteocytes in their lacunae were examined under a light microscope and rated as <50% of cells showing retraction and ≥50% of cells showing retraction. Nitric acid 3% and 5% caused ≥50% osteocyte retraction in all specimens (six out of six). Formic acid 10% caused ≥50% osteocyte retraction in four out of six specimens (66.7%), while EDTA showed ≥50% osteocyte retraction in five out of six specimens (83.3%). These findings were significant among all the agents (p-value is 0.03) [Table/Fig-14,15].

Decalcification agents	Osteocyte retraction		Total
	≥50% n (%)	<50 n (%)	
EDTA 10%	5 (83.3)	1 (16.6)	6
Formic acid 10%	4 (66.66)	2 (33.3)	6
Nitric acid 3%	6 (100)	0	6
Nitric acid 5%	6 (100)	0	6

[Table/Fig-14]: Chi-square for osteocyte retraction. The Chi-square statistic is 17.0141. The p-value is 0.03. The result is significant at p<0.05.



[Table/Fig-15]: (a) EDTA 10% ≥50% osteocyte retraction; (b) Formic acid 10% ≥50% osteocyte retraction; (c) Nitric acid ≥50%osteocyte retraction; (d) Nitric acid 5% ≥50% osteocyte retraction H&E (X40).

DISCUSSION

Microscopic examination of calcified tissues requires the preparation of decalcified sections. There are different types of chemicals used as decalcifying agents that may have adverse effects on tissues, such as swelling or shrinkage not attributed to pathological conditions [18]. In the present study, adverse effects of different decalcifying agents (EDTA 10%, Formic acid 10%, Nitric acid 3%, and Nitric acid 5%) were evaluated under microscopic examination, making it easier to choose decalcifying material with few side-effects on hard and soft-tissues.

When compared to other decalcifying agents, the specimens that underwent EDTA 10% decalcification took the longest to decalcify. This finding can be explained by the fact that EDTA is a chelating agent that binds to Ca+ in the hydroxyapatite crystal's outer layer, reducing the crystal's size during the decalcification process. Compared to the acid decalcification profile, this process could be extremely slow [19]. The decalcification time for nitric acid, regardless of its concentration, was significantly shorter. This can be attributed to the fact that nitric acid is a strong acid, which facilitates faster decalcification compared to other agents. The present study results were in accordance with studies by Bhat N et al., Choube A et al., and Sanjai K et al., who also found that in their studies, the speed of decalcification with nitric acid was the fastest, and neutral EDTA was the slowest compared to other decalcification agents [14,20,21]. Concerning formic acid, the decalcification was completed within 3.5 days because formic acid is considered a weak acid, thus taking a little more time for complete decalcification than nitric acid, which represents a strong acid [22].

The current study revealed non significant differences between the decalcification agents regarding staining intensity. Overstaining intensity was observed in more than half of the histological sections decalcified with EDTA, while most specimens decalcified with other agents showed adequate staining, especially the histological sections decalcified with 10% formic acid. These findings were supported by a previous study conducted by Gupta S et al., who found that better staining was observed with nitric acid and formic acid compared to EDTA. This difference could be attributed to the time factor, as the longer time required for complete decalcification may have more adverse effects on staining intensity [23]. In contrast, another study by Khangura A et al., found that in terms of staining quality, EDTA is the best decalcifying agent compared with other decalcifying agents [24].

Different histological principles were used for the assessment of the sections in the present study. The greatest severity of odontoblast destruction, dentin-pulp separation, irregular pulp organisation, and fraying in dentinal tubules were noted in the case of 5% nitric acid, while other decalcification agents showed relatively less destruction, especially with formic acid, with significant differences observed among the four decalcification agents regarding dentin-pulp separation and pulpal organisation. These results align with

a prior study by Prasad P and Donoghue M, which demonstrated that the application of strong acid decalcification agents may lead to damage or detachment of pulp tissue from dentin due to the rapid opening of dentinal tubules [17]. This was supported by observations of fraying in dentinal tubules and loss of odontoblast architecture in sections treated with potent acids. Similarly, another study conducted by Khangura A et al., showed that EDTA, 5% trichloroacetic acid, and 8% formic acid were the most effective agents in terms of soft-tissue attachment, shrinkage, and pulp organisation compared to other decalcification agents [24].

The maximum cementum destruction in the present study was noted in cases treated with 5% nitric acid, while destruction was negligible with another agent. In the same context, the degree of PDL separation was severe in histological sections decalcified with nitric acid in both concentrations, showing significant differences compared to other decalcification agents. These findings of the present study can also be explained by the fact that nitric acid is a strong acid that causes extensive damage and lytic effects on tissues and cells [17].

Since formic acid is a weak acid [25], and EDTA is not easily absorbed by bone tissues and has little to no effect on bone tissues due to its low affinity for calcium ions [26], these facts may help explain the observed results. Bone tissue analysis indicated that sections decalcified with formic acid and EDTA contained the largest percentage of osteoblasts around the bone trabeculae. In contrast, osteoblasts were missing in more than half of the nitric acid-decalcified histological sections, regardless of the concentration used. The majority of sections showed osteocyte retraction of ≥50%, with a few exceptions of sections treated with formic acid and EDTA, which showed a retraction of less than 50%. In summary, the choice of decalcification method can have a significant impact on the preservation of bone tissue structure and cellular components. The use of formic acid and EDTA appears to be more favourable in terms of maintaining the integrity of bone architecture compared to nitric acid.

Gupta S et al., have reported that nitric acid was shown to be the most effective due to its ability to balance tissue integrity with time, making it a reliable decalcifying agent for routine histopathology diagnosis [23], while Umbare D et al., have concluded that neutral EDTA can be suggested for tissue preservation compared with other decalcifying agents when time is not a problem, because of its capacity to preserve soft-tissue integrity and offer superior staining [27]. In contrast to the present study, where it was observed that formic acid enhanced tissue

Study No.	Author's name and year	Place of study	Sample size	Decalcifying agents compared	Parameters assessed	Conclusion
1	Bhat N et al., [20]	Not mentioned	30 extracted human premolars teeth	(10% Nitric acid, EDTA, 10% Formic acid).	Comparing the speed of action, ease of sectioning, staining properties, soft-tissue attachment and shrinking pulpal organisation.	In all aspects except time, EDTA exceeded the other groups.
2	Choube A et al., [21]	Not mentioned	80 freshly extracted human molar teeth	formic acid-formalin, formal-nitric acid, formalin EDTA,von Ebner's solution, Perenyi's fluid	Time for decalcification. Staining features, Inspect for dentin and cementum destruction, pulp organisation, and tissue artifacts.	Strong acids decalcified tissue quickly, but also impaired staining and caused extensive tissue damage. To decalcify teeth, we propose using formic acid with agitation.
3	Sanjai K et al., [14]	Not mentioned	24 human teeth (four per solution).	EDTA solution, 5% nitric acid, Perenyi's fluid, formalin-nitric acid, 5% trichloracetic acid, and 10% formic acid.	The speed of of decalcifying chemicals. Ease of sectioning, staining properties. Soft-tissue attachment and shrinking pulpal organisation.	Neutral EDTA, while being the slowest decalcifying agent, had good results for tissue integrity and staining quality.
4	Gupta S et al., [23]	The Department of Oral and Maxillofacial Pathology at Maharishi Markandeshwar College of Dental Sciences and Research, Mullana, Ambala, India.	60 human permanent teeth	10% formal nitric acid, 10% formic acid, 10% nitric acid,8% potassium formate+8% formic acid, and (EDTA).	Time of decalcification, maintain dentinal structure, clarify dentinal tubules, pulpal organisation, preservation of the odontoblastic layer, staining properties.	Nitric acid was shown to be the most effective decalcifying agent due to its ability to balance tissue integrity and time.
5	Khangura A et al., [24]	The Department of oral and maxillofacial pathology, New Delhi, India)	30 human permanent teeth (5 teeth in each solution).	8% formic acid, formalin-nitric acid,5% nitric acid, 5% trichloroacetic acid, Perenyi's fluid, and (EDTA).	Decalcification speed, ease of sectioning, staining characteristics, soft-tissue integrity (attachment, shrinkage, and pulp organisation).	5% trichloroacetic acid was shown to be the most effective decalcifying agent balancing tissue integrity and time.
6	Prasad P and Donoghue M [17]	Not mentioned	48 rat mandibular bone samples with molar teeth	Perenyi's fluid, EDTA, 10% formal nitric acid, 8% formal nitric acid, 10% formic acid, and 8% formic acid	Time for decalcification, ease of sectioning, staining properties, osteoblasts line the bone trabeculae, osteocytes retraction within the lacunae, cementum destruction, PDL sepration, dentin destruction, and separating pulp from dentin	EDTA was the most effective decalcifying agent available. However, given time constraints, formal nitric acid is recommended.
7	Umbare D et al., [27]	PIMS Loni Rural Dental College's Department of Oral Pathology and Microbiology.	50 premolar teeth	5% (EDTA), 10% formic acid, contains 5% Trichoraticectic acid, 5% nitric acid, and 5% formalin-nitric acid.	Decalcification rates, ease of sectioning, dental tissues structure, and staining properties	The formalin-nitric acid solution seems to establish a fair balance between speed and tissue preservation.
	Present study	Department of Oral Diagnosis/College of Dentistry/University of Baghdad	A total of 24 molar teeth with their surrounding alveolar bone	10% EDTA, 3% nitric acid, 5% nitric acid, and 10% formic acid	Duration of the decalcification process, staining intensity, pulp-dentin separation, pulp organisation, dentin and cementum destruction, PDL separation, osteoblasts trabeculae, osteocyte retraction	10% formic acid emerged as the most efficient decalcifying solution, ensuring rapid decalcification with favourable cellular and tissue architecture.

[Table/Fig-16]: Previous studies related to the present study [14,17,20,21,23,24,27].

preservation and staining quality within an appropriate decalcification timeframe. Similar studies from the literature have been tabulated in [Table/Fig-16] [14,17,20,21,23,24,27].

Limitation(s)

The study focused on histological evaluation, specifically tissue structure preservation. However, the compatibility of the decalcifying agents with subsequent analytical methods, such as immunohistochemistry or molecular analysis, was not explored. While there is a restricted range of decalcifying agents, there are various other decalcifying chemicals available that could be evaluated for their effects on tissues.

CONCLUSION(S)

Given the potent nature of nitric acid, regardless of concentration, and the prolonged decalcification process associated with EDTA both led to notable alterations in cellular and tissue architecture. However, the present study's findings highlight formic acid as the superior decalcifying solution. It not only achieved swift decalcification but also demonstrated promising preservation of cellular and tissue morphology and architectural integrity. In conclusion, the optimal choice of a decalcifying agent should prioritise a delicate balance between preserving tissue integrity and minimising decalcification duration.

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