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Natural Sweeteners: An Alternative to Ethanol in Oral Exfoliative Cytology

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ABSTRACT

Background: Fixation is one of the most important and primary steps for tissue or smear processing for microscopic examination. It helps us to maintain and preserve the consistent morphological features by preventing the autolysis, bacterial putrefaction of the tissue/ smear. For Cytology, Ethanol is considered as "Gold Standard". Ethanol though an efficient cytological fixative, has few disadvantages such as it is bio-hazardous, expensive, flammable, volatile. Thus, there is a need to identify an alternate and eco-friendly replacement of ethanol.

Aims: To evaluate and compare the fixative properties of Honey, sugar and jaggery with ethanol in oral cytology smear.

Methodology: Comparative analytical study was carried out with 20 healthy individuals of similar age group without any oral pathologies and informed consent was taken. 4 oral smears obtained from buccal mucosa of each individual (n=20) by gentle scrapping. Slides were prepared and coded. Fixation done using: Bio Fix (Ethanol 95%), Sugar Solution (30%), Honey (20%), Jaggery solution (30%). Staining was done with Papanicolaou stain. Three Pathologists (observers), blinded for the fixative, evaluated the slides for five parameters i.e. Cell morphology, nuclear staining, cytoplasmic staining, clarity of staining and uniformity of staining.

Results: The result shows that Honey has better fixative property as compared to jaggery and sugar and was statistically significant to that of ethanol, while jaggery showed minimum fixative properties.

Conclusions: The present study shows that honey demonstrated comparable fixative properties to ethanol. So, honey can be used as an alternative to ethanol for cytological fixation.

Keywords: *cytological diagnosis, exfoliative cytology, cyto-techniques, oral exfoliative cytology, natural sweeteners, cell preservation, cytological fixatives.*

1. INTRODUCTION

In the realm of cytology, preserving cellular morphology is paramount for accurate diagnosis and analysis.[1] Over the years, various fixatives have been employed to immobilize cells and maintain their structural integrity for microscopic examination. Among these, ethanol has stood as a cornerstone due to its effectiveness in preserving cellular architecture.[2] However, recent endeavours have sought alternative fixatives, particularly those derived from natural sources, to address concerns regarding the toxicity and availability of conventional fixatives.[3] With this interest, substances like honey, jaggery, and sugar have emerged as potential candidates, boasting not only fixative properties but also intrinsic therapeutic benefits.[4]

Ethanol, a traditional fixative, penetrates cells rapidly, coagulating proteins and preserving cellular structures.[3] Its widespread use in cytology laboratories underscores its reliability; it is subjected to pilferage, expensive, flammable and volatile and may cause irritation to skin and eyes on prolonged exposure. In recent classification of human carcinogen, IARC Classified ethanol as class I human carcinogen.[5] All these concerns have prompted the exploration of alternative options. Honey, a natural product revered for its antimicrobial and wound-healing properties, presents itself as a promising substitute.[4] Rich in sugars, enzymes, and antioxidants, honey possesses inherent fixative properties that may rival those of ethanol, while also offering additional therapeutic benefits to the cells under examination.[6]

Traditionally, Sugar have been used to preserve food, and the ability of sugar to attract water (hygroscopicity) also influence the texture. Sugar, a ubiquitous dietary component, completes the trio of natural fixatives under scrutiny. Despite its simplicity, sugar's ability to dehydrate cells and inhibit microbial growth has long been recognized in food preservation.[7] This desiccating effect, coupled with its capacity to form hydrogen bonds with cellular proteins, imparts fixative properties that warrant investigation in cytological contexts.

Similarly, jaggery, a traditional sweetener derived from sugarcane or palm sap, bears resemblance to honey in its chemical composition. Jaggery exhibits potential as a fixative agent owing to its ability to denature proteins and stabilize cellular structures.[8] Moreover, its availability and affordability make it an attractive alternative for resource-limited settings, where conventional fixatives may be scarce or prohibitively expensive.

As we embark on this comparative analysis, it is imperative to acknowledge the multifaceted nature of cytology and the intricate balance between fixation, preservation, and interpretation of cellular specimens.While ethanol has long reigned supreme as the gold standard fixative, the quest for alternatives is fuelled by a desire for safer, more sustainable, ecofriendly and cost-effective practices that align with the principles of holistic healthcare.

In this study, we endeavour to unravel the fixative potential of honey, jaggery, and sugar in oral cytology smears, juxtaposing their efficacy and practical feasibility against the benchmark set by ethanol for cytological smears. The oral cavity, with its diverse cellular compositions and susceptibility to various pathologies, serves as an ideal landscape for such investigations. By scrutinizing the ability of these natural substances to immobilize oral epithelial cells, we aim to shed light on their potential as alternative fixatives, offering insights into their efficacy, and practical utility in cytological applications.

Methodology

The comparative analytical study was conducted in the Dept. of Oral & Maxillofacial Pathology and Microbiology, Sri Aurobindo College of Dentistry, Indore, Madhya Pradesh.This study was carried out among healthy volunteers giving informed consent, aged more than 18 years, without any oral lesions and excluding those having any adverse oral habit.

The sample size was estimated on the basis of the study conducted by Pandiar et al.[8],which shows the accuracy of honey as 96% for nuclear staining, so considering the highest accuracy of the staining and taking power of study as 80% at 95% confidence interval, the formula applied was,

$$n = \frac{Z^2 - P(1-P)}{D^2} = 58.98$$

Where: Z=1.96, P=96% or 0.96, D=0.05.

So total sample size was estimated as 60 smears, which was equally divided into 3 experimental groups and for comparison, similar number of control samples of standard (Ethanol) was also taken. Thus, total number of smears made were 80.

Preparation of fixative solutions

30% aqueous Sugar solution (w/v) was made by dissolving 30 g of refined sugar (bought from the local market of Indore) in 70 mL of distilled water.

20% aqueous honey solution (v/v) was formed by mixing 20 mL of honey (“Dabur Honey, Dabur India ltd, Solan, India”) in 80 mL of distilled water.

30% aqueous jaggery solution (w/v) was formed by dissolving 30 g of jaggery (bought from the local market of Indore) in 70 mL of distilled water.

The solutions formed were then filtered through filter paper ofand kept in a transparent plastic spray bottleto be used for further procedure.

The fresh solutions were prepared a day prior to the sample collection to be used within a week and kept refrigerated to avoid mould formation.

Ethanol (95% Biofix) was used as standard for the purpose of comparison.

Figure 1: Material used as fixatives

Figure 1: Material used as fixatives: (A) Bio-fix {95% ethanol}, (B) Sugar, (C) Honey and (D) Jaggery

Sample collection and evaluation

Four smears made from buccal mucosa of each individual by gentle scrapping using wooden spatula and divided in four group, one smear in each group from an individual. Slides were coded in groups as: A-Ethanol, B-Sugar, C- Honey, D-Jaggery.

Fixation is done by spraying solution on smear in each group as Group A- Bio Fix (Ethanol 95%), Group, B- Sugar Solution (30%), Group C- Honey solution (20%), Group D- Jaggery solution (30%) and left to air dry for 15-20 minutes.

The slides were then washed in running tap water for about 20-30 sec and staining is done using the Rapid Papanicolaou stain kit.

The cytoplasmic and nuclear details were evaluated using following parameters i.e. Cell morphology, nuclear staining, cytoplasmic staining, clarity of staining and uniformity of staining. Three Pathologists (observers) who were blinded for the fixative used in each group observed the slides and recorded the results for said parameters by scoring them as poor (score 1), average (score 2), good (score 3) and excellent (score 4).Median of the scores given by all the observers for all the parameters was taken as final score to be analysed.

2. RESULTS

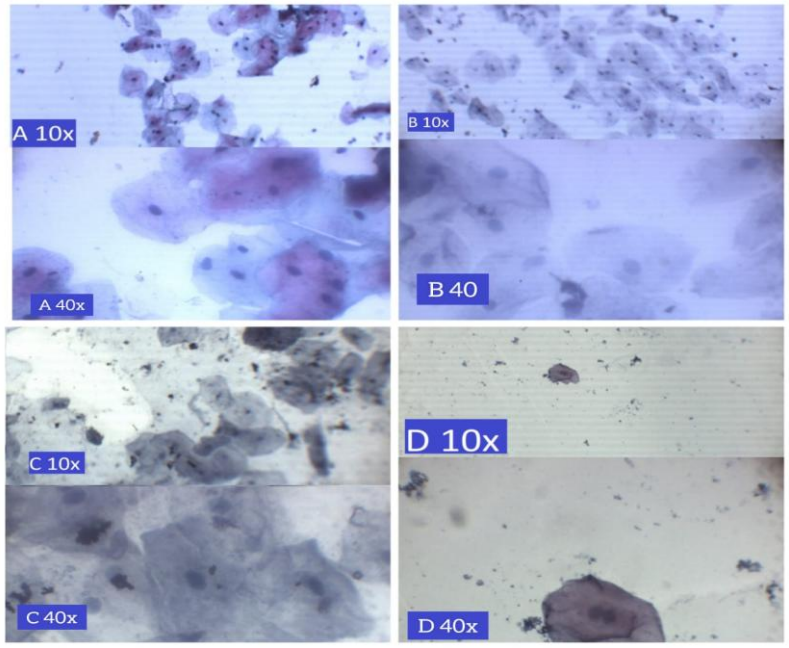

Figure 2: Stained smear after fixation by A- Ethanol, B-Sugar, C-Honey, D-Jaggery

Table 1: Fixative properties scored by observers for five different parameters

Property	Quality	Fixative			
		Ethanol n=20 (%)	Sugar n=20 (%)	Honey n=20 (%)	Jaggery n=20 (%)
Nuclear Staining	Poor	0(0)	1(5)	0(0)	1(5)
	Average	0(0)	0(0)	3(15)	4(20)
	Good	8(40)	5(25)	5(25)	8(40)
	Excellent	12(60)	14(70)	12(60)	7(35)
Cytoplasmic Staining	Poor	0(0)	0(0)	0(0)	1(5)
	Average	0(0)	7(35)	2(10)	11(55)
	Good	10(50)	4(20)	7(35)	5(5)
	Excellent	10(50)	9(45)	11(55)	3(15)
Uniformity of cell	Poor	0(0)	2(10)	0(0)	1(5)
	Average	0(0)	4(20)	4(20)	15(75)
	Good	7(35)	12(60)	6(30)	4(20)
	Excellent	13(65)	2(10)	10(50)	0(0)
Morphology of Cell	Poor	0(0)	1(5)	0(0)	5(25)
	Average	0(0)	0(0)	3(15)	5(25)
	Good	2(10)	10(50)	1(5)	8(40)
	Excellent	18(90)	9(45)	16(80)	2(10)
Clarity of Cell	Poor	0(0)	0(0)	0(0)	2(10)
	Average	0(0)	0(0)	9(45)	10(50)
	Good	6(30)	15(75)	2(10)	6(30)
	Excellent	14(70)	5(25)	9(45)	2(10)

Table 1 shows the quality of the different properties of all four fixatives. Results show that properties of nuclear staining were found to be highest in Sugar fixed slides with 70% of excellent scoring, followed by Ethanol and Honey which is 60% while only 35% of jaggery fixed slides shows excellent nuclear staining by sugar among all four fixatives. Honey and Ethanol and Sugar shows 55%, 50% and 45% excellent score for cytoplasmic staining while jaggery shows only 15% excellent score.The uniformity of cell was found with the excellent score of 65% and 50% in ethanol and honey fixed slides respectively while in jaggery fixed slide it shows zero score, 60% of the sugar fixed slides showed good score.90% and 80% of ethanol and honey fixed slides showed excellent score for Morphology of cell, while 90% of sugar fixed slide showed a total of good and excellent score. Clarity of cell was observed to be 70 % excellent in ethanol fixed and 45% in honey fixed slides but 75% of sugar fixed slide showed only good score. Overall Jaggery showed least excellent scoring in all the parameters taken for the fixative properties.

Table 2: Mean quality of each fixative for given five parameters

Parameter	Fixative used	Mean	SD	F value	P value
Nuclear staining	Ethanol	3.60	0.50	2.47	0.08
	Sugar	3.60	0.75		
	Honey	3.45	0.76		
	Jaggery	3.05	0.89		
Cytoplasmic Staining	Ethanol	3.50	0.51	7.54	0.01*
	Sugar	3.10	0.91		
	Honey	3.45	0.69		
	Jaggery	2.50	0.83		
Uniformity of Cell	Ethanol	3.65	0.49	19.88	0.01*
	Sugar	2.70	0.80		
	Honey	3.30	0.80		
	Jaggery	2.15	0.49		
Morphology of Cells	Ethanol	3.90	0.31	16.95	0.01*
	Sugar	3.35	0.75		
	Honey	3.65	0.75		
	Jaggery	2.35	0.99		
Clarity of Cell	Ethanol	3.70	0.47	11.53	0.01*
	Sugar	3.25	0.44		
	Honey	3.00	0.97		
	Jaggery	2.40	0.82		

Test applied- Chi square test, *p value ≤ 0.05-statistically significant.

Table 2 depicts the mean quality score of all parameters for each fixative. There was no significant difference found among the fixatives for nuclear staining. For cytoplasmic staining, there was statistically significant difference with p value of 0.01 was found among groups, having higher mean score of ethanol (3.50±0.51) and honey (3.45±0.69). Mean score of ethanol (3.65±0.49) and honey (3.30±0.80) were also found higher for the uniformity of cells and the result shows statistically significant difference among all the groups with p value of 0.01. Similarly for morphology of the cells also shows significantly higher(p=0.01)in ethanol and honey than other fixatives with the mean value of 3.90 and 3.65 respectively. While for the clarity of the cells, the mean score of ethanol(3.70) and sugar(3.25) and honey(3.00) are comparable but are significantly higher than jaggery. Among all the groups Jaggery showed least mean scores for all the parameters.

3. DISCUSSION

Over the years, various fixatives have been employed to immobilize cells and maintain their structural integrity for microscopic examination. Among these, ethanol has stood as a cornerstone due to its effectiveness in preserving cellular architecture, thus aiding in cytological assessment and diagnosis.[9]Ethanol, though an efficient cytological fixative, but because of its bio-hazardous nature, there is a need to identify an eco-friendly alternate to alcohol fixative.This study was conducted to determine the fixative properties of three natural sweeteners viz, honey, sugar and jaggery and compare

themwith the standard fixative- ethanol.

The present study showed that there is no significant difference between the staining properties of honey and ethanol. Jaggery showed the least significant results as compared to sugar and honey.Results of the present study are similar to the study done by Singh et al.[3] and Pandiar et al.,[8] who analysed the efficacy of ethanol and 20% unprocessed honey as a cyto-fixative and concluded that honey could be safely used as a substitute for ethanol. Priyadarshi et al.[10] and Ishaq et al.,[11] compared honey with alcohol and concluded that honey shows excellent fixative properties and can be used as a fixative, which is comparable to the present study. Lalwani et al.[12] and Sabarinath etal.[4] compared honey with formalin and showed no statistically significant difference between honey and formalin samples for both nuclear details and cytoplasmic staining. They suggested that both processed honey and unprocessed honey can be used as an alternative to formalin, which is similar to our study as the present study also shows no statistically significant difference between the staining properties of honey and ethanol. Patil et al.[13]compared the tissue fixation ability of 20% honey, 20% sugar syrup, and 30% jaggery syrup with that of 10% buffered formalin and done staining with haematoxylin and eosin (H and E) stain. They observed that the tissue fixed with jaggery had good overall morphology, nuclear and cytoplasmic details, and staining quality with a clearly discernible cellular outline as compared to other fixatives. However, in our study, we observed that jaggery showed the least fixative properties compared to ethanol and other natural sweeteners used in the study.Imran et al.[14] in their study observed that tissue fixed with sugar syrup showed good overall morphology, nuclear and cellular outline, compared to formalin fixatives except for cytoplasmic details and staining quality. In our study, we found that cells fixed with sugar solution showed a good nuclear stain, and overall morphology is also maintained. Similarly, tissue fixed with honey showed good nuclear and staining quality but poor overall morphology, cytoplasmic, and cellular outline in their study. On the contrary, our study results show that the properties of honey are almost similar to ethanol.

The present study explores the naturaland eco-friendly alternatives (honey, sugar, jaggery) to ethanol, addressing current concerns over ethanol's carcinogenicity and hazards. Use of **blinded observers** (pathologists) reduces bias and improves the **objectivity** of scoring.

Although the sample size is statistically justified, the **sample size of 20 per group** may still limit generalizability, thus, larger multicentric studies are required for more detail. The present study used **only normal exfoliated oral cells**, which limits the understanding of fixative performance in **diseased or atypical cytology**. The honey, sugar, and jaggery were all bought from local sources and not standardized. Variability in the composition of natural product could affect reproducibility. Solutions were prepared fresh and refrigerated for short-term use. **Shelf-life, microbial contamination, or stability over time** were not evaluated.

4. CONCLUSIONS

The results of present study conclude that honey can be used as an alternative to ethanol as its fixative properties are similar to that of ethanol for the fixation of oral exfoliative cytological smears. Sugar and jaggery can also be used but they have less comparable results than honey. In the present study jaggery was found to be the least suitable fixative to be used for cytology. To conclude we can say that natural sweeteners being cheap and easily available can be used as an alternate to ethanol for fixation of smears in the remote areas for the screening of community at field.

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