Original Article

Use of jaggery and honey as adjunctive cytological fixatives to ethanol for oral smears

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Abstract

Background: Ethanol has satisfactorily been used as cyto-fixative. Owing to its limitations, pathologists have always searched for new fixatives. The present study was conducted to evaluate the efficacy of two natural sweeteners as cyto-fixatives. An attempt has also been made to understand the underlying mechanism by which these fixatives fix the oral mucosal cells.

Materials and Methods: Three smears were collected from 25 healthy volunteers. One smear was fixed in ethanol and the other two in 20% aqueous honey solution and 30% aqueous jaggery solution for 15–30 min followed by Papanicolaou staining. Slides were evaluated for nuclear staining, cytoplasmic staining, cell morphology, clarity of staining and uniformity of staining randomly irrespective of the fixatives. Chi-square test and Bonferroni *post hoc* test were done using SPSS software. $P < 0.05$ was considered to be statistically significant. In addition, cytological fixatives were analyzed for the pH and amount of reducing sugars.

Results: It was found that for all the characteristics studied, no statistically significant difference was seen between the three fixatives. The pH of the both tested fixatives remained acidic even after 1 week. The amount of reducing sugars in 20% aqueous honey solution and 30% aqueous jaggery solution was 19.3 g/100 mL and 2.07 g/100 mL, respectively.

Conclusion: Both the test fixatives gave results equivalent to ethanol and thus can be used as alternative fixatives for oral smears. It is proposed that 20% aqueous honey and 30% aqueous jaggery fix the oral smears satisfactorily in a mechanism akin to ethanol by coagulating and denaturing proteins.

Keywords: Cytological fixatives, honey, jaggery, pH, reducing sugars

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INTRODUCTION

Many important systemic disorders including hematological, dermatological, endocrinal or even rheumatological diseases manifest in the oral cavity, and thus, the oral cavity can be considered as a window to the body. Owing to the rapid turnover rate of oral mucosal cells, the exfoliated cells may have a valuable role in diagnosis of aforementioned disorders as reflected by cytomorphological and nucleomorphological variations in the exfoliated cells. Exfoliative cytology is based on the monitoring the mucosal exfoliated cells which wither through natural or artificial means.[1]

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Ninety-five percent ethanol is the standard cytological fixative used in many laboratories. Commercially available ethanol is expensive and not freely available in some institutions. Pathologists have always been exploring for an ideal cytological fixative. Methanol and recently natural sweeteners such as honey have been effectively used as cytological fixatives.[2,3]

Recently, Patil *et al.* used 20% honey solution and 30% jaggery solution as routine tissue fixative.[4,5] It was found that the jaggery was comparable to formalin in tissue preservation followed by honey. They also proposed a possible mechanism by which jaggery and honey fix tissues. It was opined that fructose present in these natural sweeteners at low pH breaks down to aldehydes and cross-links with the amino acids present in the tissue. Most recently Singh *et al.* used 20% unprocessed honey solution for cytological specimen from oral mucosa and compared the results with ethanol. They found that both ethanol- and honey-fixed smears were at par with each other and concluded that honey could be safely used as a substitute to ethanol.[3]

Honey and jaggery have been used since centuries as sweetening and medicinal agents. Honey primarily contains sugar and water. Sugar accounts for 95%–99% of honey dry matter. Majority of these are simple sugars, fructose (38.2%) and glucose (31.3%). It has been shown to have an antimicrobial action against a broad spectrum of bacteria and fungi.[6] Honey has also been used as an agent for preventing autolysis and putrefaction.[3] Akin to honey, jaggery contains high content of reducing sugars, which can be exploited for tissue fixation. The micronutrients present in jaggery have antitoxic and anticarcinogenic effects.[7]

Both honey and jaggery are being used in pathology laboratories for tissue fixation, but their role in cytopathology is still at experimental level. The search in the English literature for an eco-friendly cytological fixative, alternative to ethanol, has resulted in dearth of information. Thirty percent aqueous jaggery solution as a cytological fixative has not been used so far; neither any attempt has been made on the determination of the amount of reducing sugars in 20% aqueous honey solution and 30% aqueous jaggery solution. Hence, in the present study, we used 30% jaggery solution as a cytological fixative and compared it with 20% honey and 95% ethanol. In addition, we intended to detect the exact composition of reducing sugars in 20% honey and 30% jaggery solutions so that an explanation for their mechanism of action can be unraveled.

MATERIALS AND METHODS

Our study population encompassed 25 healthy volunteers who attended the Department of Conservative Dentistry, Faculty of Dental Sciences, Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi. Approval of the Departmental Research Committee was obtained before commencement of the study.

Initially, the natural sweeteners (honey and jaggery) were analyzed for the pH and percentage of reducing sugars in the Department of Chemistry, Institute of Science, BHU. The oral smears were then stained by conventional Papanicolaou staining in the Department of Pathology, IMS, BHU.

Preparation of fixative solutions

a. 95% ethanol (v/v): 95 mL of ethanol mixed with 5 mL of distilled water
b. 20% aqueous honey solution (v/v): 20 mL honey (Dabur Honey, Dabur India Limited, Solan, India) was dissolved in 80 mL of distilled water
c. 30% aqueous jaggery solution (w/v): 30 g of jaggery (obtained from the local market of Varanasi) was dissolved in 70 mL of distilled water. The solution thus obtained was filtered through a filter paper and used for further procedure.

Analysis of reducing sugars (using Lane–Eynon method)

There are several ways to determine the sugar content of the food stuff such as chromatographic, volumetric, colorimetric, gravimetric and enzymatic methods. In addition, physical methods such as polarimetry and refractive index methods are also available. Volumetric method, mainly Lane–Eynon method, is often the most convenient method for determining reducing sugars. This is a short and rapid method and often the most accurate method for the estimation of reducing sugars. It is based on the determination of the volume of a test solution required to reduce completely a known volume of alkaline copper reagent. The end point is indicated by the use of an internal indicator, methylene blue.

Equipment and chemicals

Heater, burette, sensitive scale, Fehling I (69.278 g copper sulfate was dissolved in distilled water and made up to 1 L), Fehling II (100 g sodium hydroxide and 346 g sodium potassium tartrate was dissolved in distilled water and made up to 1 L), 5.0% glucose solution and methylene blue indicator (1.0% methylene blue solution was prepared by using distilled water) were used [Figure 1a].

Procedure

All the above-mentioned solutions were prepared 2 days before the experiment. Honey sample was diluted down to 3% concentration, and the burette was filled with that. 5 mL Fehling I and 5 mL Fehling II solutions were added consecutively to a conical flask and 10 mL distilled water was added to a glass beaker. The flasks were then heated on a hot plate to boil, and the water was transferred to the first flask [Figure 1b]. A 5–6 drops of methylene blue were added as indicator. The flask was titrated till observing brick red color [Figure 1c and d]. The operation was repeated using 5% glucose syrup instead of sample as standard.

Data and calculations

Amount of glucose (g) = (S1 × 5)/1000

S1: The consumed volume (ml) of glucose (standard)

Amount of reducing sugars in honey sample (g) = (Amount of glucose × 100)/S2

S2: The consumed volume (ml) of honey sample.

Similar procedure was repeated using 3% aqueous jaggery solution. Thus, the concentration of reducing sugars in 20% aqueous honey and 30% aqueous jaggery solutions was mathematically determined.

pH

The pH of 20% aqueous honey solution and 30% aqueous jaggery solution was detected using pH meter (ISO-Tech System, ITS-201, pH meter, India) and monitored for a week.

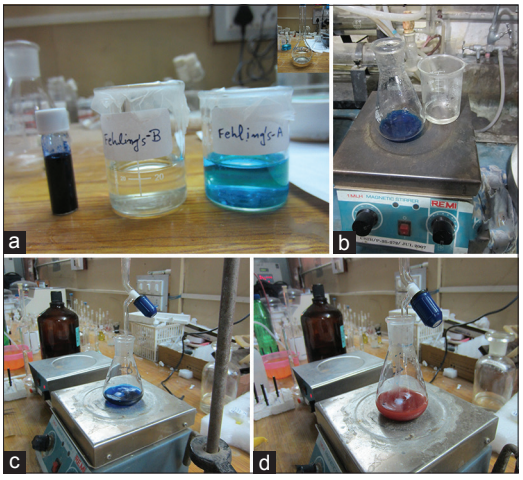

Figure 1: Armamentarium and procedure. (a) Reagents used: Methylene blue indicator, Fehling I and Fehling II solution and 5% glucose solution (inset). (b) Heating the solutions on heater. (c and d) Titration until brick red color is obtained

Collection of smears

Before obtaining buccal smears, patients were asked to rinse their mouth with water. Buccal cells were collected using a metallic cement spatula. Three smears will be collected from each subject. One smear was fixed in ethanol and the other two in 20% aqueous honey solution and 30% aqueous jaggery solution for 15–30 min. The slides were then washed in tap water for about 30 s, following which they were subjected to the conventional Papanicolaou staining procedure.

Papanicolaou staining

Slides were treated with increasing grades of alcohol and stained with Harris Hematoxylin for 2 min. The slides were then washed in running tap water for 8–10 min. Acid differentiation was done in acid alcohol followed by washing in running water. The slides were then dipped for 30 s each in 90% alcohol and 70% alcohol, respectively. The next step involved staining in OG 6 for 2 min followed by two changes in 90% alcohol. Slides were then stained with EA 36 for 2 min followed by increasing grades of alcohol. The slides were finally cleared and mounted in dextrene polystyrene xylene.

Evaluation of slides

The cytoplasmic and nuclear details were scored for 50 cells in each slide. Two pathologists evaluated the slides for nuclear staining, cytoplasmic staining, cell morphology, clarity of staining and uniformity of staining randomly irrespective of the fixative. The slides were categorized into poor (score 0), intermediate (score 1) and good (score 2). The data were entered in MS Office Excel spread sheet and statistically compared using the Pearson Chi-square test followed by Bonferroni *post hoc* test. A $P < 0.05$ was considered as statistically significant. For statistical analysis, SPSS software (IBM Analytics) was used.

RESULTS

1. Amount of reducing sugars in 20% aqueous honey and 30% aqueous jaggery solution: The detailed quantification of reducing sugars in 20% aqueous honey solution and 30% aqueous jaggery solution is shown in Tables 1 and 2, respectively

Table 1: Quantification of reducing sugars in 20% aqueous honey solution (by Lane-Eynon method)

Test solution	Amount of 5% glucose consumed (S1)	Amount of 3% honey solution consumed (S2)	Amount of glucose; S1×5/1000	Amount of reducing sugars in 3% honey sample (amount of glucose × 100/S2)	Amount of reducing sugars (20% aqueous honey solution)
3% aqueous honey solution	8.7 mL	1.5 mL	0.0435 g	2.9 g	19.3 g/100 mL

2. The pH of 20% honey and 30% jaggery solution: The pH of 20% aqueous honey solution and 30% aqueous jaggery solution as monitored over 6 days is shown in Table 3
3. Evaluation of Papanicolaou stained smears [Figures 2-5]
    - Nuclear staining: It was found that all of the honey-fixed samples showed good staining as compared to 96% of jaggery-fixed and 88% of ethanol-fixed smears [Figure 6]. However, the difference between these fixatives was not statistically significant (Chi-square value: 5.197, $P = 0.268$; df: 4)
    - Cytoplasmic staining: Among all the tested fixatives, smears fixed in ethanol showed the highest percent of good cytoplasmic staining (72%) as compared to 68% percent of honey-fixed smears and 64% of jaggery-fixed smears. About 24% ethanol-fixed, 28% honey-fixed and 32% jaggery-fixed samples showed intermediate cytoplasmic staining [Figure 7]. On statistical comparison, the $P = 0.982$ implying that there was no statistically significant difference between these three fixatives (Chi-square value: 0.403; df: 4)
    - Cellular morphology: The highest percentage of poor cellular morphology was seen in jaggery-fixed smears (12%) whereas honey-fixed smears showed the best preservation of cellular morphology among the three fixatives (52%) [Figure 8]. $P = 0.725$

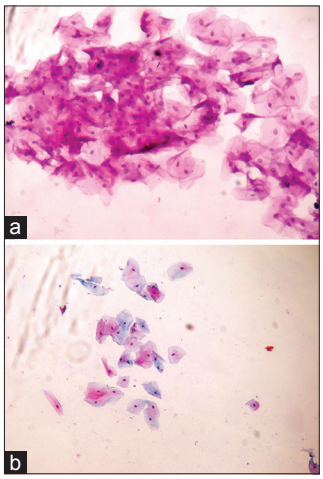

Figure 2: Photomicrograph showing ethanol-fixed smears (PAP stain; a and b - ×40)

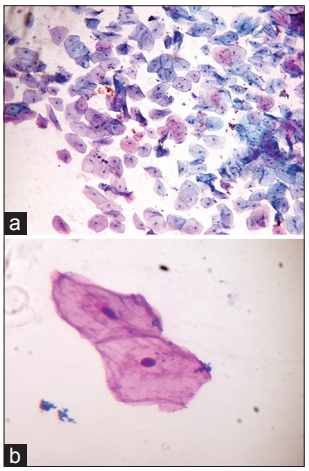

Figure 3: (a and b) Photomicrograph showing honey-fixed smears (PAP stain; [a] - ×40, [b] - ×400)

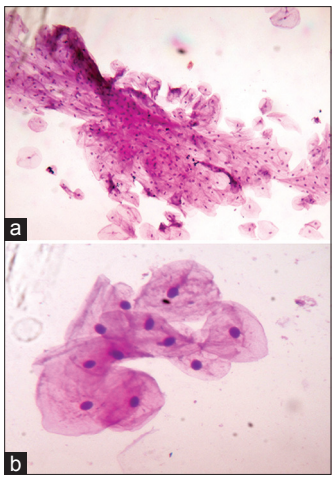

Figure 4: (a and b) Photomicrograph showing jaggery-fixed smears (PAP stain; [a] - ×40, [b] - ×400)

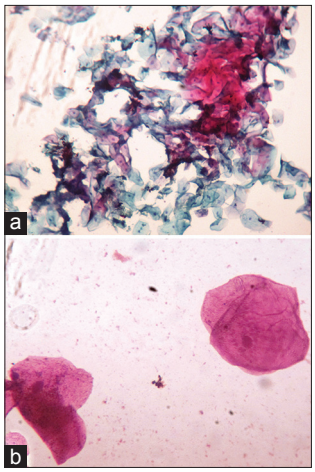

Figure 5: Artefacts common to all fixatives. (a) Extreme distortion of cell morphology (PAP, ×40). (b) Eosinophilic nuclei (PAP, ×400)

Table 2: Quantification of reducing sugars in 30% aqueous jaggery solution (by Lane-Eynon method)

Test solution	Amount of 5% glucose consumed (S1)	Amount of 3% jaggery solution consumed (S2)	Amount of glucose; S1×5/1000	Amount of reducing sugars in 3% jaggery sample (amount of glucose × 100/S2)	Amount of reducing sugars (30% aqueous jaggery solution)
3% aqueous jaggery solution	8.7 mL	21 mL	0.0435 g	0.207 g	2.07 g/100 mL

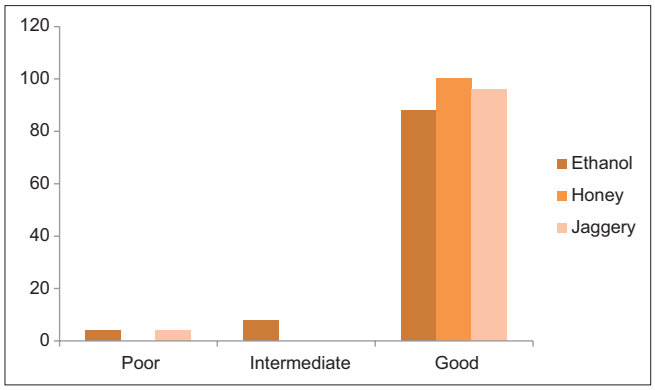

Figure 6: Correlation of nuclear staining among ethanol-fixed, honey-fixed and jaggery-fixed smears (in percentage)

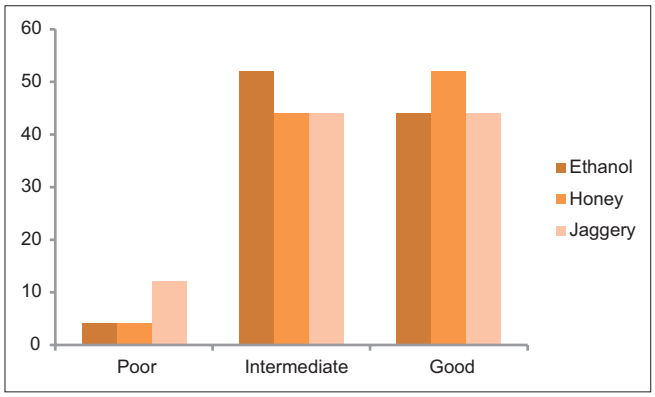

Figure 8: Correlation of cellular morphology among ethanol-fixed, honey-fixed and jaggery-fixed smears (in percentage)

was observed, and thus, there was no statistical difference between these fixatives (Chi-square value: 2.057; df: 4)

- Clarity of staining: Figure 9 shows percentage distribution of quality of clarity of staining among the three fixatives. Honey-fixed samples showed overall best clarity, but there was no statistically significant difference (Chi-square value: 2.784; $P = 0.595$; df: 4)
- Uniformity of staining: About 80% of honey-fixed smears showed good overall uniformity in staining followed by jaggery-fixed smears (60%) and ethanol-fixed smears (44%). The overall detailed percentage is shown in Figure 10. There was no statistically significant difference among the three fixatives regarding this characteristic (Chi-square value: 9.009; $P = 0.061$; df: 4).

4. Multiple comparisons: Bonferroni *post hoc* analysis showed that when the three fixatives were individually compared to each other for the characteristics, there was no statistically significant difference between groups for nuclear staining, cytoplasmic staining, cell morphology and clarity of staining. However, it was found that uniformity of staining was better in smears fixed with honey as compared to ethanol, and the results were statistically significant ($P = 0.042$). Ethanol and jaggery ($P = 1.000$) and honey and jaggery ($P = 0.291$) were at par in maintaining uniform staining.

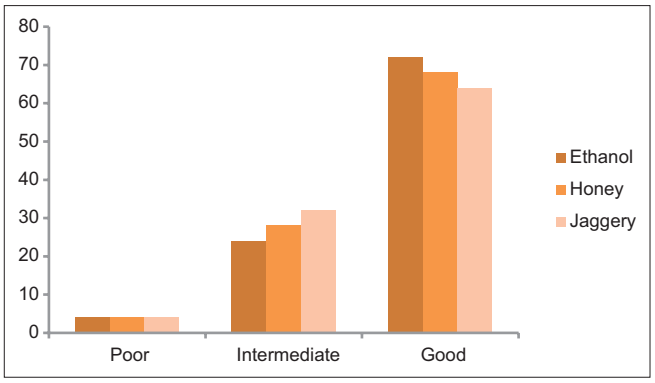

Figure 7: Correlation of cytoplasmic staining among ethanol-fixed, honey-fixed and jaggery-fixed smears (in percentage)

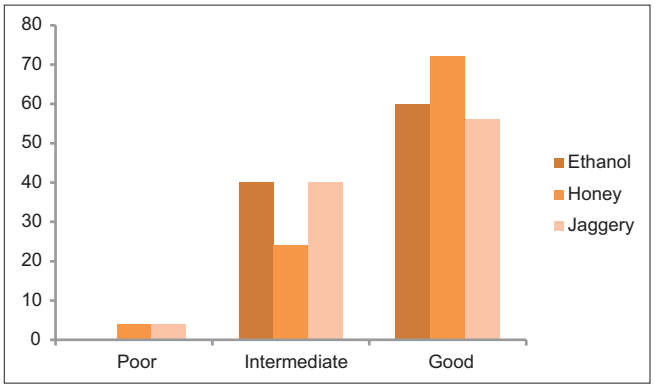

Figure 9: Correlation of clarity of staining among ethanol-fixed, honey-fixed and jaggery-fixed smears (in percentage)

Table 3: Monitoring of pH in 20% aqueous honey and 30% honey solution

Day	pH of 20% honey solution	pH of 30% honey solution
1	4.64	5.44
2	5.5	5.5
3	5.5	6
4	6	6.5 (molds appeared)
5	6 (molds appeared)	6.7
6	6	6.7

DISCUSSION

Fixation of smears is a step of utmost significance in cytopathology laboratories as unfixed smears always yield results which are impossible to discern. It may be difficult to read a well-fixed well-stained smear, but it is impossible to read a poor slide. We pathologists have always quested for a fixative which is better than customary fixatives with least harmful effects and best results. Ethanol has been serving satisfactorily for years; however, it has its own limitations.

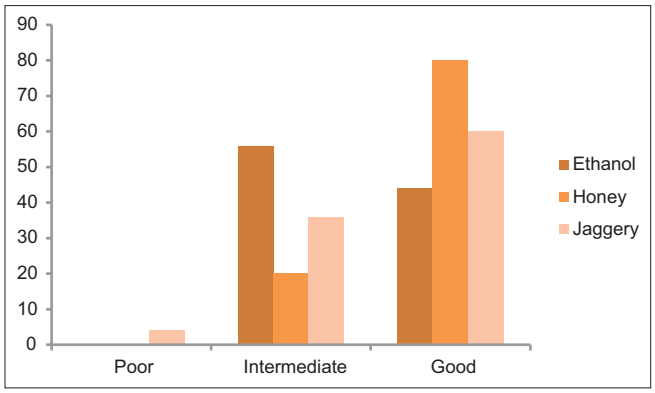

Figure 10: Correlation of uniformity of staining among ethanol-fixed, honey-fixed and jaggery-fixed smears (in percentage)

It is subjected to pilferage, is expensive, carcinogenic, evaporates easily and is not freely available.[3] Thus, the aim of this study was to find an alternative(s) which give(s) result equivalent to ethanol if not better.

In the present study, it was found that for all the characteristics studied, and there was no statistically significant difference between the three fixatives. Seen individually, smears stained with honey showed the highest percentage of score 2 (good). All the smears fixed in honey showed good nuclear staining while 68% smears showed good cytoplasmic staining. Well-preserved cell morphology was seen in 52% smears, a score which was higher than ethanol- and jaggery-fixed smears (44% each). Good overall clarity and uniformity of staining by honey were seen in 72% and 80% of smears, respectively. Interestingly, it was found that uniformity of staining was better in smears fixed with honey as compared to ethanol, and the results were statistically significant ($P = 0.042$). Ethanol and jaggery ($P = 1.000$) and Honey and jaggery ($P = 0.291$) were at par in maintaining uniform staining. Singh *et al.* in their study analogized the potency of ethanol and honey.[3] Akin to our findings, they also found no statistically significant difference between ethanol and honey solutions. They thus inferred that honey and ethanol fixed smears were equivalent in efficacy and proposed that honey could be used as a substitute to ethanol.

Ten percent honey was used in a study by Sabarinath *et al.* who compared 10% formalin and with 10% honey solution.[8] In contrast, Patil *et al.* in their studies used honey and jaggery for tissue fixation at 20% and 30% concentration, respectively.[4,5,9] The concentration of honey and jaggery solution selected in the present study was similar to various studies stated above.

Patil *et al.* proposed that fructose present in honey and jaggery at acidic pH breaks down to aldehydes which cross-link with tissue amino acids similar to the action of formaldehyde.[4] In the present study, we also endeavored to explore the underlying mechanism of fixation of honey and jaggery solutions.

It would be a blind attempt to try various fixatives unless the composition and mechanism are clearly understood. In quest of the solution, the exact composition of reducing sugars (believed to be involved in fixation process) and pH was determined. Reducing sugars were determined by Lane–Eynon method which is the most convenient volumetric method for determining reducing sugars. In addition, it is deemed to be a short, rapid and an accurate method for quantification of reducing sugars. The amount of reducing sugars in 20% honey solution and 30% jaggery solution was 19.3 g/100 mL and 2.07 g/100 mL, respectively.

The pH of 20% honey and 30% jaggery solution was monitored over 1 week using an electronic pH meter. Honey is characteristically acidic with a pH of between 3.2 and 4.5, which serves to inhibit the growth of many animal pathogens.[10] Hydrogen peroxide and gluconic acid produced enzymatically serve to preserve honey and on dilution, the activity increases by a factor of 2500–50,000, thus giving "slow-release" antiseptics at a level, which is antibacterial but not tissue damage.[11] We found that at baseline (day 1), diluted solution of honey at 20% concentration also had acidic pH (4.64). Similar to diluted honey solution, 30% jaggery solution had an acidic pH (5.44). On the 6th day, the pH rose to 6 for 20% honey solution and 6.7 for 30% jaggery solution. This rise in pH can be explained on the fact that all carbohydrates over a period of time breakdown to alcohol; a mechanism exploited in brewery industry since ages.

Glucose and fructose have the same molecular formula ($C_6H_{12}O_6$), but glucose has a six member ring and fructose has a five member ring structure. Interconversions in the structure of fructose and glucose can occur enzymatically. At an acidic pH, one molecule of glucose breaks down into two molecules each of ethanol and carbon dioxide as shown below.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

As depicted in the results section, fungal growth appeared on the 5th day in honey solution and 4th day in jaggery solution. Fungi supply the enzymes invertase, maltase and zymase. Intrinsic enzymes of honey such as invertase and maltase may also assist in the process. The enzyme invertase hydrolyses sucrose to glucose and fructose, and the enzyme zymase converts glucose and fructose to ethanol. As the

alcohol concentration rises, the fungi are unable to survive since a concentration of more than 12% kills the fungi. Thus, unlike other staining solutions where appearance of fungal molds is considered to be detrimental, it may be advantageous in honey and jaggery fixative solutions. Interestingly, fungal growth did not produce hindrance with clarity and uniformity of staining possibly due to self-killing mechanism of molds at alkaline pH. Fixatives were discarded on the 8th day not because of molds but due to the pungent smell yielded during fermentation of sugars.

Thus, we conclude that honey and jaggery fix the tissue in alcohol like manner rather than formaldehyde. Ethanol is considered to be coagulant that denatures proteins. It replaces water in the tissue environment disrupting hydrophobic and hydrogen bonding thus exposing the internal hydrophobic groups of proteins and altering their tertiary structure and their solubility in water.

The results of the present study showed that no statistically significant difference in the efficacy of these fixatives possibly due to similar mechanism of fixation. We thus opine that honey and jaggery solutions are at par in efficacy to ethanol and may be used as alternatives to ethanol in certain conditions such as private practice and rural areas where ethanol may not be readily available.

The major drawbacks seen with these fixatives were that both attracted insects and had a short shelf life. However, the advantages overweighed the disadvantages. Both the fixatives, i.e., honey and jaggery, are biocompatible, cheap, readily available, nonvolatile, not subjected to pilferage and are easy to discard.

CONCLUSION

Ethanol is an excellent cyto-fixative, but natural sweeteners such as 20% honey and 30% jaggery have given equivalent results to ethanol and thus can be used as alternative fixatives for oral smears if not the substitutes. It is proposed that 20% honey containing 19.3 g/100 mL reducing sugars and 30% jaggery containing 2.07 g/100 mL reducing sugars fix the oral smears satisfactorily in a mechanism akin to ethanol by coagulating and denaturing proteins.

Acknowledgment

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Nil.

Conflicts of interest

There are no conflicts of interest.

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