

## Original Research Article

# A comparative study on the efficacy of honey and ethanol as cytological fixatives

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**Received:** 10 March 2017

**Accepted:** 06 April 2017

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### ABSTRACT

**Background:** In cytological fixatives prior to Papanicolaou staining smears are fixed mostly in alcohol or any alcohol based special purpose fixative. Alcohols are costly, hazardous and also there is a problem of disposal. Here, a very cheap and easily available alternate for fixative was being used. The objective was to describe the efficacy of 20% honey as a cytological fixative in comparison to ethanol.

**Methods:** The study was done on buccal smears from students of Department of MLT, Government Medical College, Thiruvananthapuram, Kerala, India for a period of six months after ethical approval. Two buccal smears were collected from either cheek using wooden spatula/blunt end of the Ayers spatula. One of the smear is fixed in ethanol (95%) and the other smear is fixed in honey (20%). The slides were scored based on the assessment parameters: nuclear staining, cytoplasmic staining, cell morphology, clarity of staining and uniformity of staining.

**Results:** The study was done on buccal smears, the sample were smeared and fixed onto slides using two fixatives- 95% ethanol and 20% honey. Both reported good quality staining feature, preservation of morphology and a crisp nuclear and cytoplasmic staining. It has also added advantage of being nontoxic, economical, non-flammable, non-hazardous, no problems associated with license of purchase, reduces overall turnaround time and is easy to handle without any sort of irritation.

**Conclusions:** Thus, the overall study shows that honey has got a fixative action, which can be implemented soon in the industry.

**Keywords:** Ethanol, Fixative, Honey, Papanicolaou staining

### INTRODUCTION

Cells are the building block of all living things. Groups of these cells, called tissues unite to perform specific function. Microscopic study of individual cell in a smear is called cytology and study of tissue is called histology.<sup>1</sup> After the removal of samples from a patient, a series of processes must take place in order to ensure the final microscope slides are of diagnostic quality. The quality of the structural preservation of tissue components is

determined by the choice of reagent and exposure time to that reagent during processing.<sup>2</sup>

Fixation is the first and foremost step required for the proper diagnosis. Most tissues are fixed before they are examined microscopically. For carrying out the upcoming techniques, it is essential that fixation is effective and that the appropriate fixative is used.<sup>3</sup> Basic aim in any field of life science is to utilize eco-friendly chemicals which are non-toxic, less bio-

hazardous, and are economical. Alcohols are an inevitable part of cytology laboratory; where various grades have been utilized as fixative. The quench for alternatives never ends; so that alternative for fixative also. Therefore, any alternative fixative that minimizes the use of bio-hazardous substitutes, reduces the staining time and does not compromise the staining quality will be very valuable in diagnostic cytology. The present study used a natural fixative- honey instead of chemicals in order to prevent the deleterious effects of the chemical fixatives.

**Objective**

To describe the efficacy of 20% honey as a cytological fixative in comparison to ethanol.

**METHODS**

The buccal smears from students of Department of MLT, Government Medical College, Thiruvananthapuram, Kerala, India for a period of six months were taken for the study. Two buccal smears were collected from either cheek using wooden spatula/blunt end of the Ayers spatula.

One of the smear is fixed in ethanol (95%) and the conventional method of Papanicolaou staining is done. And the other smear is fixed in honey (20%) and the modified method of Papanicolaou staining which omits the first steps of hydration before staining procedure. Smear processing and staining were done in Pathology lab, Department of MLT, Medical college, Thiruvananthapuram, Kerala, India.

**Sample size calculation**

**Formula**

Sample size calculation using the formulae;

$$N = \frac{\{z_1 - \frac{a}{2\sqrt{2p(1-p)}} + z_1 - \beta\sqrt{p_1(1-p_1)p_2(1-p_2)}\}^2}{(p_1 - p_2)^2}$$

Where,  $p = \frac{(p_1-p_2)}{2}$

- P1: Proportion in the first group
- P2: Proportion in the second group
- a: Significance level
- 1-β: Power

According to the similar study<sup>18</sup>

**Results**

- P1: 97%
- P2: 90%
- a: Significance level = 5%
- 1-β: Power =80%
- N = 194 in each group

**Inclusion criteria**

Normal and healthy buccal smears are collected.

**Exclusion criteria**

Inflamed or abnormally appearing buccal mucosal smears.

**Preparation of fixative**

- 95% Ethanol- mix 95ml Ethanol and 5ml distilled water.
- 20% Honey- mix 20ml Honey and 80ml distilled water.

**Analysis**

The smears collected were fixed in Honey and Ethanol and were analyzed for the following parameters given in the Table 1.

- Nuclear staining
- Cytoplasmic staining
- Cell morphology
- Clarity of staining
- Uniformity of staining.

**Table 1: Analysis of different parameters.**

Features	Score and criteria	
	Acceptable = 1	Unacceptable =0
Nuclear staining	Round smooth and clear membrane	Granular, disintegrated and out of focus.
Cytoplasmic staining	Intact cytoplasmic membrane and transparent membrane.	Disintegrated cytoplasmic, granular cytoplasm and out of focus.
Cell morphology	Absence of folds and maintained NC ratio	Overlapping cells folded and disintegrated.
Clarity of staining	Crispiness in staining and transparency	Obliterates the nucleus and cytoplasm.
Uniformity of staining	Uniformly stained throughout the individual cell	Stained in different shades of colour in an individual cell

## RESULTS

The present study evaluated the efficacy of Honey in comparison with ethanol as a cytological fixative for Papanicolaou staining. 194 samples were processed for each method of fixation.

### Ethanol fixed smears vs honey fixed smears

Five parameters of staining characters are evaluated for the both method. The measure of agreement kappa is being utilized to evaluate the degree of agreement.

The measure of agreement between the both method for total score was found to be 0.879 which shows a strong agreement between the two methods studied. The mean of total score obtained by the new method of fixation is 3.93 and 3.94 for ethanol fixed smears.

The five parameters used to assess were:

- Nuclear staining
- Cytoplasmic staining
- Cell morphology
- Clarity of staining
- Uniformity of staining.

### Nuclear staining

Of the 194 slides 97% reported adequate for Ethanol and 96% for Honey. About 4% were inadequate for Honey and 3% Ethanol.

**Table 2: Nuclear staining.**

Nuclear staining	Ethanol		Total	
	Inadequate	Adequate		
Honey	Inadequate	4	2	6
	Adequate	1	187	188
Total	5	189	194	

Measure of agreement Kappa = 0.719

The measure of agreement kappa is 0.719 which is strong agreement between the two methods- Honey and Ethanol fixation for Nuclear staining.

### Cytoplasmic staining

**Table 3: Cytoplasmic staining.**

Cytoplasmic staining	Ethanol		Total	
	Inadequate	Adequate		
Honey	Inadequate	8	2	10
	Adequate	3	181	184
Total	11	183	194	

Measure of agreement Kappa = 0.748

The measure of agreement kappa is 0.748 which is strong agreement between the two methods- Honey and Ethanol fixation for cytoplasmic staining.

### Cell morphology

**Table 4: Cell morphology.**

Cell morphology	Ethanol		Total	
	Inadequate	Adequate		
Honey	Inadequate	51	2	53
	Adequate	1	140	141
Total	52	142	194	

Measure of agreement Kappa = 0.961

The measure of agreement kappa is 0.961 which is strong agreement between the two methods- Honey and Ethanol fixation for cell morphology.

Of the 194 slides 73% reported adequate for Ethanol and 72% for Honey. About 28% were inadequate for Honey and 27% Ethanol.

### Clarity of staining

**Table 5: Clarity of staining.**

Clarity of staining	Ethanol		Total	
	Inadequate	Adequate		
Honey	Inadequate	71	1	72
	Adequate	2	120	122
Total	73	121	194	

Measure of agreement Kappa = 0.967

The measure of agreement kappa is 0.967 which is strong agreement between the two methods- Honey and Ethanol fixation for clarity of staining. Of the 194 slides 62% reported adequate for Ethanol and 61% for Honey. About 39% were inadequate for Honey and 38% Ethanol.

### Uniformity of staining

**Table 6: Uniformity of staining.**

Uniformity of staining	Ethanol		Total	
	Inadequate	Adequate		
Honey	Inadequate	63	3	66
	Adequate	2	126	128
Total	65	129	194	

Measure of agreement Kappa = 0.942

The measure of agreement kappa is 0.942 which is strong agreement between the two methods- Honey and Ethanol fixation for uniformity of staining.

Of the 194 slides 66% reported adequate for Ethanol and 64% for Honey. About 36% were inadequate for Honey and 34% Ethanol.

**Total staining quality (score)**

**Table 7: Total staining quality.**

Total score		Ethanol			Total
		Bad	Good	Excellent	
Honey	Bad	12	0	1	13
	Good	1	36	4	41
	Excellent	0	4	136	140
Total		13	40	141	194

Measure of agreement Kappa = 0.879

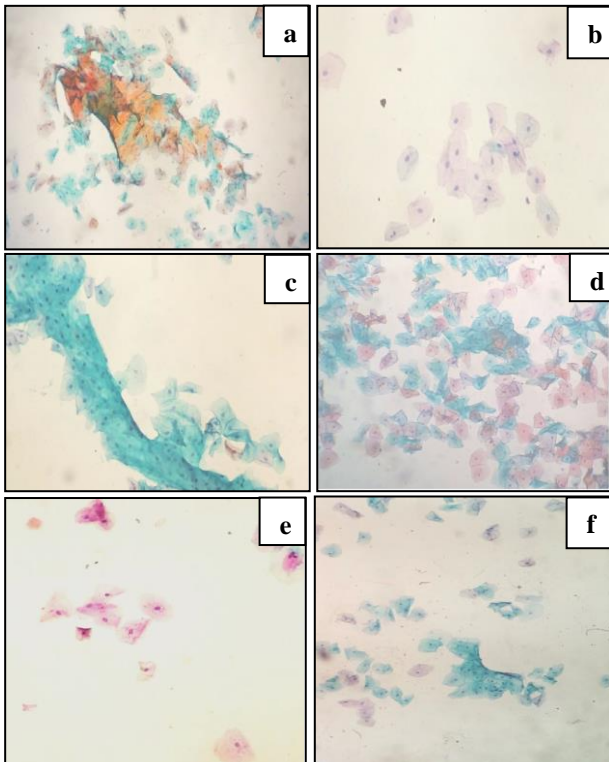
The measure of agreement kappa is 0.879 which is strong agreement between the two methods- Honey and Ethanol fixation for total score.

**Overall staining quality**

**Table 8: Overall staining quality.**

	N	Total score		t-value	p-value
		Mean	SD		
Ethanol	194	3.94	1.02	0.200	0.842
Honey	194	3.93	1.03		

Mean value=3.93 for Honey fixed smear; Mean value=3.94 for ethanol fixed smear.



**Figure 1: Photomicrograph of pap stained both ethanol and 20% honey fixed smears. a, b and c - Squamous cells, Superficial cells and intermediate cells in honey fixed smear; d, e and f - Squamous cells, Superficial cells and intermediate cells in ethanol fixed smear.**

**DISCUSSION**

Various fixatives have been employed in both cytology and histopathology. Apart from stain in Papanicolaou staining technique, other component is fixative, graded alcohols and xylene. These chemicals are used in intermediate steps of hydration, dehydration, clearing and mounting during staining. The lacunae however continue to persist in this old-age procedure are the cost containment, toxicity, problem of disposal of the hazardous chemicals, alcohols, xylene and pollution of working environment.

**Fixation**

Fixation of cell preparations is a common procedure for the best possible preservation of cell components after removal from the tissue of origin.

The important factors involved in fixation are:

**Hydrogen ion concentration**

- Temperature
- Penetration
- Osmolarity
- Concentration
- Duration.

In present study we have compared the efficacy of 20% honey as a cytological fixative to ethanol.

The fixative of choice for gynecologic and other smear preparations was the one recommended by Dr. George N. Papanicolaou, namely, a solution of equal parts of ether and 95% ethyl alcohol. Subsequently, it has been necessary to abandon this original and excellent fixative because ether presents a fire hazard. 95% ethyl alcohol (ethanol) is now employed as a best fixative by most laboratories, with excellent results. Smears should remain in the 95% ethyl alcohol fixative for a minimum of 15 minutes prior to staining.

To obtain ethanol without federal taxation, a license is required. Ethanol is classified by the International Agency for Research on Cancer (IARC) as causing cancer in humans following ingestion.<sup>4</sup> Skin exposure from spillages of ethanol can cause burning and stinging. Eye exposure to ethanol can also cause burning and stinging.<sup>5</sup>

Honey is the natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants.<sup>6</sup> Honey is a mixture of sugars and other compounds. The various properties of honey include antioxidant, antimicrobial and anti-autolytic effects. It has the quality of penetrating the deepest tissue and can prevent autolysis and

putrefaction.<sup>7</sup> Honey has been found to prevent autolysis as tissues put in it for up to 30 days did not show any sign of putrefaction and autolysis. The tissue hardening property makes it similar in action to fixatives which act by hardening the tissues.<sup>8</sup>

The antibacterial effect of honey depends on its osmotic effect (high sugar and low water content), acidity, hydrogen peroxide formed by enzymatic reaction and phytochemical factors. Most unprocessed honey, when diluted slowly, generates hydrogen peroxide which has antibacterial properties.<sup>9</sup> The present study was undertaken to assess and document the efficacy of processed honey and unprocessed honey as an alternative fixative for histological sections. Honey was collected from wild bee colonies, or from domesticated beehives.<sup>10</sup> Used as food, in beverages and Medical uses are in cough, wounds and burns.<sup>11,12</sup> Indicator of quality for honey is Agmark certification mark which is legally enforced in India by the Agricultural produce (Grading and Marking) Act of 1937.<sup>13</sup>

To go organic is a theme of the present day; an attempt was made to explore the natural substance honey as a substitute for fixation of smears. With an added benefit of honey being eco-friendly, easily available, cost effective, nontoxic and non-inflammable, it can also be used as an effective alternative.<sup>14</sup> Main findings and problems encountered were:

#### ***Loss of materials***

The initial problem encountered with the study was the "loss of material" during processing the smears. Almost all of the slides reported scanty cellularity and were not suitable for diagnosis. Later the sample smeared on to the slide was dried for 1-2 minutes and then put into the newer fixative (diluted honey)<sup>4</sup>. These smears were then checked for cellularity, which were adequate for diagnosis or reporting.

#### ***Growth of moulds***

The second problem encountered with the study was growth of moulds in the fixative (diluted honey). After three to four days of storage, it was observed that a layer of moulds as a film over the newer fixative. The slides that were fixed in this fixative showed several mould forms. Even when a newly diluted fixative was kept the same problem hindered the study. Later thymol crystals were preferred to prevent the growth of moulds.<sup>15</sup>

#### ***Use of adhesives***

Even when the loss of cellularity was a major problem associated with the study, any of the adhesives recommended were not preferred to maintain cellularity. Duration of fewer seconds to minutes was preferred to overcome the difficulty.

The use of adhesives or coating of adhesives may also lead to the shedding of squamous cells from the skin of fingers which may alter the diagnosis. Thus, all these factors preferred to dry the slides to recover all these problems.<sup>16</sup>

#### ***Absence of drying artifact***

Time duration of less than two minutes was preferred before fixation in the newer fixative. This minimum period of air drying of these wet smears showed no visible alteration in the morphological features or staining property of the cells.<sup>17</sup> Thus, the drying of smears was utilized to prevent cell loss during fixation in newer fixative (diluted honey) and also to avoid the use of adhesives.

#### ***N/C ratio***

Nuclear-cytoplasmic ratio is preferred with this newer fixative technique and this method can also be implemented for morphometric studies.

#### ***Fixation for 15 minutes***

Time duration of 15 minutes was preferred in order to parallel with the conventional fixative used for the study. 95% ethanol is preferred as the conventional fixative.<sup>2</sup> This time was enough for the newer fixative (20% honey diluted with distilled water) to fix the cells without altering its morphology and staining property.

#### ***Preferring buccal smear for the study***

Buccal smears were preferred for the study because of these characters.<sup>1</sup>

- Easy to obtain
- Abundant material
- Repeatability
- Non-invasive procedure

#### ***Advantages of buccal smears over cervical smears***

Cervical smears were not preferred because the smears were difficult to obtain and the case can't be called for repeating the sample as required for the study. If the sample collected is inadequate for reporting, it would also result in dilemma for what being done.<sup>9</sup>

#### ***Vaporization***

The rate of getting vaporization is less for honey when compared with that of ethanol, which is an advantage for the newer fixative preferred.<sup>17</sup>

#### ***Inflammability***

The newer fixative preferred is not flammable and thus being a better comparable quality with that of ethanol or any alcohol containing fixative preferred routinely.<sup>10</sup>



## Viscosity

For this study 20% diluted honey is preferred which losses almost a better percentage of its viscosity which makes its easy removal from slide. The hydration step followed by removes almost all remnants from the slides before the staining steps starts.

## Color of fixative

The newer fixative is colored but it doesn't impart any color to the cells they fix.<sup>18</sup> This was also adding a point on honey as a fixative.

## CONCLUSION

The objective of this study is to compare the efficacy of Ethanol with Honey as a cytological fixative. This study utilized the easy availability of buccal smears from the students of Department of MLT. Here the measure of agreement of our new method with that of the conventional method is very high. The statistical evaluation says that this newer method of fixation can be implemented after proper standardization.

Honey fixed smears gave a high measure of agreement with that of Ethanol fixed smears for

- Nuclear staining character
- Cytoplasmic staining character
- Cell morphology
- Clarity of staining
- Uniformity of staining

Thus, the overall study shows that the Honey has got a fixative action, which can be implemented soon in the industry.

This newer method of fixation is technician friendly for use which is less irritant and does not require any license for purchase and is economical comparably. A largescale production of honey and making its availability feasible are further questions faced.

As the whole world is moving to become holistic this method of fixation without any chemicals will become significant in the industry.

*Funding: No funding sources*

*Conflict of interest: None declared*

*Ethical approval: The study was approved by the institutional ethics committee*

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**Cite this article as:** Sona M, Preethamol S. A comparative study on the efficacy of honey and ethanol as cytological fixatives. *Int J Adv Med* 2017;4:762-7.