ORIGINAL ARTICLE

Evaluation of Distilled White Vinegar and Apple Cider Vinegar as Alternatives to 95% Ethanol for Buccal Cell Fixation in Cytological Evaluation

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ABSTRACT

Introduction: Vinegar has been explored as an alternative fixative for buccal cells to substitute 95% ethanol in the Papanicolaou staining protocol due to ethanol's health risks after long-term exposure. The acetic acid in vinegar can stabilize cells by preventing nucleic acid loss and precipitating DNA. This study evaluates the effectiveness of distilled white vinegar and apple cider vinegar compared to 95% ethanol as fixatives. **Materials and Method:** Accordingly, buccal cells were fixed using distilled white vinegar, apple cider vinegar, and 95% ethanol. Cytomorphological characteristics, including nucleus size, cytoplasm size, nuclear-cytoplasmic ratio, and colour intensity were measured using Toupview software and analyzed with One-way ANOVA and independent t-tests. A qualitative assessment was performed by 10 observers using a scoring system to evaluate the buccal cells. **Results:** The findings indicated no significant differences in cytomorphological characteristics between buccal cells fixed with distilled white vinegar and 95% ethanol ($p > 0.05$), whereas apple cider vinegar caused significant changes in the characteristics ($p < 0.05$). Buccal cells fixed with distilled white vinegar show comparable results to those fixed with 95% ethanol. In contrast, buccal cells fixed with apple cider vinegar do not produce comparable results to those fixed with 95% ethanol. Distilled white vinegar had moderate agreement (Fleiss' Kappa = 0.524), while apple cider vinegar had poor agreement (Fleiss' Kappa = -0.017). **Conclusion:** The study concluded that distilled white vinegar is a suitable alternative to 95% ethanol for buccal cell fixation, offering similar effectiveness in preserving cell morphology and colour.
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Keywords: Distilled white vinegar, apple cider vinegar, 95% ethanol, fixatives, and cytomorphological characteristics

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INTRODUCTION

Cytological studies especially in oral cancer diagnosis have given considerable attention to the analysis of buccal cells (1). Buccal cells are obtained from the cheek's inner lining by non-invasive procedures (2, 3). Fixation is needed to preserve these buccal cells before undergoing staining and observation to obtain an accurate evaluation and analysis. Due to its ability to dehydrate and denaturize proteins, ethanol particularly in concentrations of 70% to 100% has been used extensively as a standard fixative. However, ethanol has risks to one's health and safety, including flammability, poisonous fumes, and other potential workplace hazards (4, 5). Researchers have conducted several studies to find other fixatives that can replace ethanol and they come out with studies about methanol, honey, and jaggery which in the results show their ability to become cell fixatives but the efficacy is different compared to ethanol (6, 7).

The search for an alternative to 95% ethanol as a cell fixative continues, with vinegar becoming the interest of the investigation. Vinegar made from fruits or other natural sources, is a typical household product consisting of acetic acid and water which can be used as a multipurpose ingredient or agent. Examples of commercially available vinegar include distilled white vinegar, apple cider vinegar, balsam vinegar, wine vinegar, and more. Several studies have proven that vinegar can become a preservative, especially for food, and also a disinfectant because it has some properties to prevent fungal and bacterial growth (8, 9). One of the compounds in vinegar, acetic acid, also known as

ethanoic acid, is produced naturally by fermentation. Vinegar may replace ethanol in cell fixation since acetic acid can help to prevent nucleic acid loss and stabilize the nucleus of cells by DNA precipitation (10). Acetic acid in vinegar may have similar fixative characteristics as ethanol due to its acidity and capacity to denature proteins. Even though acetic acid can preserve the nucleus based on past studies, further studies are needed to investigate the ability of acetic acid to preserve other cellular morphology of buccal cells.

MATERIALS AND METHODS

Samples

Healthy students from the Faculty of Health Science, UiTM Puncak Alam volunteered to collect their buccal cell samples. The students were asked to rinse their mouths with tap water several times followed by 0.9% normal saline to remove any food particles before collecting buccal cells. After that, the inside of their cheek was scrapped firmly using a wooden stick then the collected buccal cells were smeared onto microscope glass slides properly. 12 buccal cell samples were collected from three volunteers, and four were collected from each volunteer. Then, the buccal cells were fixed with different fixatives which were absolute distilled white vinegar, absolute apple cider vinegar, 95% ethanol (positive control), and unfixed (negative control) for 30 minutes. The distilled white vinegar and apple cider vinegar used were from Heinz brand and were purchased from Jaya Grocer supermarket. The ethics were approved by the Faculty Review Ethics Committee (FERC) [Reference No: FERC/FSK/MR/2024/00207].

Evaluation of nucleus size, cytoplasm size, and nuclear-cytoplasmic ratio at different time intervals after fixation (Unstained smear)

The unfixed, fixed with distilled white vinegar, fixed with apple cider, and fixed with 95% ethanol slides which were not stained were observed under the microscope. Photomicrographs were taken at 40x magnification and were transferred to a computer. An average of 5 cells with defined outlines were selected per subject for image analysis. The software used for image analysis was ToupView software by Hangzhou ToupTek Photonics Co., Ltd. Buccal cells were measured stepwise from the upper left corner to the right and then down to avoid errors in the measurement of the cells. The cells were measured for their nucleus size, cytoplasm size, and nuclear-cytoplasmic (N/C) ratio. Computer-based measurement tool in the software was used to calculate the cellular and nucleus area by outlining the cellular and nucleus outlines using a digitalized cursor. The nucleus area represented the nucleus size meanwhile the difference between the cellular and nucleus areas was calculated to find the cytoplasm area representing the cytoplasm size. Next, the nuclear-cytoplasmic ratio was calculated using the formula, N/C ratio = nucleus size/cytoplasm size (11). The observations were done at different time intervals which were 1 hour, 7 hours, and 24 hours after fixation. The unfixed buccal cells were the negative control meanwhile the buccal cells fixed with 95% ethanol were the positive control. The data were statistically analyzed through One-way ANOVA by using Statistical Package for Social Sciences (SPSS).

Colour intensity analysis (Stained smear)

The buccal cells fixed with distilled white vinegar, apple cider vinegar, and 95% ethanol were stained by the Papanicolaou staining protocol, and the colour intensity of the nucleus and cytoplasm of buccal cells was measured accordingly by analyzing the image observed under the microscope using ToupView software. The data then were analyzed by using One-way ANOVA.

Evaluation by observers

After staining, the slides were observed and evaluated under a light microscope at 10x and 40x magnification by ten observers. Two lecturers, one staff and seven students from the Medical Laboratory Department evaluated the slides. A scoring system based on the modified features was used to independently evaluate the buccal cells using five different features given by Singh et al. (2015). The statistical analysis was conducted using the Fleiss' Kappa test.

RESULTS

Evaluation of nucleus size, cytoplasm size, and nuclear-cytoplasmic ratio at different time intervals after fixation (Unstained smear)

The nucleus size, cytoplasm size, and nuclear-cytoplasmic ratio at 1 hour, 7 hours, and 24 hours after fixation were measured by using Toupview software. The experiment was replicated three times to find the mean and standard deviation of each cytomorphological characteristic. The data were recorded and tabulated in Table I.

The One-Way ANOVA indicates that there were significant differences between the mean nucleus size at 1 hour after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F(3, 8) = 8.86$, $p = 0.006$). Subsequent post-hoc analysis (Dunnett t) suggested that the mean nucleus size of cells fixed with apple cider vinegar ($M = 12.57$, $SD = 1.18$) and unfixed cells ($M = 13.67$, $SD = 1.49$) were significantly higher than the mean nucleus size of cells fixed with 95% ethanol ($M = 10.00$, $SD = 0.98$). There was no significant difference between the mean nucleus size of cells fixed with distilled white vinegar compared to the mean nucleus size of cells fixed with 95% ethanol. For the mean cytoplasm size, the One-Way ANOVA Welch test shows that there were significant differences between the mean cytoplasm size at 1 hour after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F(3, 3.98) = 23.65$, $p = 0.005$). Post-hoc

Table I: Mean and standard deviation of the nucleus size, cytoplasm size, and N/C ratio at different time intervals after fixation

Variables	Fixatives	1-hour (n=3) Mean (SD)	7-hour (n=3) Mean (SD)	24-hour (n=3) Mean (SD)
Nucleus size	95% Ethanol	10.00 (0.98)	10.36 (1.04)	10.37 (1.58)
	Distilled White Vinegar	9.99 (0.32)	10.02 (1.01)	9.94 (1.67)
	Apple Cider Vinegar	12.57 (1.18)	12.36 (0.69)	12.48 (1.38)
	Unfixed	13.67 (1.49)	13.29 (0.52)	11.81 (1.77)
Cytoplasm size	95% Ethanol	390.61 (73.63)	402.47 (13.38)	414.07 (9.14)
	Distilled White Vinegar	374.88 (6.79)	376.08 (56.07)	399.66 (49.08)
	Apple Cider Vinegar	321.99 (8.70)	310.15 (14.60)	384.79 (47.52)
	Unfixed	488.85 (46.00)	503.35 (57.41)	508.64 (49.04)
N/C ratio	95% Ethanol	0.026 (0.005)	0.026 (0.004)	0.025 (0.004)
	Distilled White Vinegar	0.029 (0.006)	0.027 (0.001)	0.026 (0.005)
	Apple Cider Vinegar	0.042 (0.006)	0.041 (0.003)	0.034 (0.007)
	Unfixed	0.029 (0.004)	0.027 (0.002)	0.024 (0.004)

SD = Standard deviation

analysis (Dunnett t) suggested that the mean cytoplasm size of cells fixed with distilled white vinegar, fixed with apple cider vinegar, and unfixed cells was not statistically different compared to the mean cytoplasm size of cells fixed with 95% ethanol. The p-value was significant because there was a significant difference between the mean cytoplasm size of unfixed cells compared to the mean cytoplasm size of cells fixed with apple cider vinegar based on the Scheffe test. For the mean N/C ratio, the One-Way ANOVA shows that there were significant differences between the mean N/C ratio at 1 hour after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F (3, 8) = 7.50$, $p = 0.010$). Dunnett t (post-hoc analysis) suggested that the mean N/C ratio of cells fixed with apple cider vinegar ($M = 0.042$, $SD = 0.006$) was significantly higher than the mean N/C ratio of cells fixed with 95% ethanol ($M = 0.026$, $SD = 0.005$). There was no significant difference between the mean N/C ratio of cells fixed with distilled white vinegar and unfixed cells compared to the mean N/C ratio of cells fixed with 95% ethanol.

Next, the One-Way ANOVA indicates that there were significant differences between the mean nucleus size at 7 hours after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F (3, 8) = 10.49$, $p = 0.004$). Subsequent post-hoc analysis (Dunnett t) suggested that the mean nucleus size of cells fixed with apple cider vinegar ($M = 12.36$, $SD = 0.69$) and unfixed cells ($M = 13.29$, $SD = 0.52$) were significantly higher than the mean nucleus size of cells fixed with 95% ethanol ($M = 10.36$, $SD = 1.04$). There was no significant difference between the mean nucleus size of cells fixed with distilled white vinegar compared to the mean nucleus size of cells fixed with 95% ethanol. For the mean cytoplasm size, the One-Way ANOVA Welch test shows that there was a significant difference between the mean cytoplasm size at 7 hours after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F (3, 4.08) = 20.96$, $p = 0.006$). Post-hoc analysis (Dunnett t) was conducted and suggested that the mean cytoplasm size of unfixed cells ($M = 503.35$, $SD = 57.41$) was significantly higher than the mean cytoplasm size of cells fixed with 95% ethanol ($M = 402.47$, $SD = 13.38$). There was no significant difference between the mean cytoplasm size of cells fixed with distilled white vinegar and apple cider vinegar compared to the mean cytoplasm size of cells fixed with 95% ethanol. For the mean N/C ratio, the One-Way ANOVA also shows that there was a significant difference between the mean N/C ratio at 7 hours after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F (3, 8) = 24.03$, $p < 0.001$). Dunnett t (post-hoc analysis) suggested that the mean N/C ratio between cells fixed with apple cider vinegar ($M = 0.041$, $SD = 0.003$) was significantly higher than the mean N/C ratio of cells fixed with 95% ethanol ($M = 0.026$, $SD = 0.004$). There was no significant difference between the mean N/C ratio of cells fixed with distilled white vinegar and unfixed cells compared to the mean N/C ratio of cells fixed with 95% ethanol.

Furthermore, the One-Way ANOVA indicates that there were significant differences between the mean nucleus size at 7 hours after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F (3, 8) = 10.49$, $p = 0.004$). Subsequent post-hoc analysis (Dunnett t) suggested that the mean nucleus size of cells fixed with apple cider vinegar ($M = 12.36$, $SD = 0.69$) and unfixed cells ($M = 13.29$, $SD = 0.52$) were significantly higher than the mean nucleus size of cells fixed with 95% ethanol ($M = 10.36$, $SD = 1.04$). There was no significant difference between the mean nucleus size of cells fixed with distilled white vinegar compared to the mean nucleus size of cells fixed with 95% ethanol. For the mean cytoplasm size, the One-Way ANOVA Welch test shows that there was a significant difference between the mean cytoplasm size at 7 hours after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F (3, 4.08) = 20.96$, $p = 0.006$). Post-hoc analysis (Dunnett t) was conducted and suggested that the mean cytoplasm size of unfixed cells ($M = 503.35$, $SD = 57.41$) was significantly higher than the mean cytoplasm

size of cells fixed with 95% ethanol (M = 402.47, SD = 13.38). There was no significant difference between the mean cytoplasm size of cells fixed with distilled white vinegar and apple cider vinegar compared to the mean cytoplasm size of cells fixed with 95% ethanol. For the mean N/C ratio, the One-Way ANOVA also shows that there was a significant difference between the mean N/C ratio at 7 hours after fixation between cells fixed with 95% ethanol, distilled white vinegar, apple cider vinegar, and unfixed cells ($F(3, 8) = 24.03$, $p < 0.001$). Dunnett t (post-hoc analysis) suggested that the mean N/C ratio between cells fixed with apple cider vinegar (M = 0.041, SD = 0.003) was significantly higher than the mean N/C ratio of cells fixed with 95% ethanol (M = 0.026, SD = 0.004). There was no significant difference between the mean N/C ratio of cells fixed with distilled white vinegar and unfixed cells compared to the mean N/C ratio of cells fixed with 95% ethanol.

Colour intensity analysis

The nucleus and cytoplasm colour intensity of the buccal cells fixed with distilled white vinegar, apple cider vinegar, and 95% ethanol for N1, N2, and N3 was measured as tabulated in Table II. Next, One-Way ANOVA tests were performed to determine if there were any statistically significant differences in the mean colour intensity of the nucleus and cytoplasm fixed with 95% ethanol, distilled white vinegar, and apple cider vinegar.

The One-Way ANOVA indicates a significant difference between the mean colour intensity of the nucleus fixed with 95% ethanol, distilled white vinegar, and apple cider vinegar ($F(2, 6) = 6.89$, $p = 0.028$). Subsequent post-hoc analysis (Scheffe) suggested that the mean colour intensity of the nucleus fixed with distilled white vinegar (M = 127.05, SD = 23.04) was significantly lower than the mean colour intensity of the nucleus fixed with apple cider vinegar (M = 188.34, SD = 9.05). However, there was no significant difference between the mean colour intensity of the nucleus fixed with distilled white vinegar and apple cider vinegar compared to the mean colour intensity of the nucleus fixed with 95% ethanol.

In addition, the One-Way ANOVA also shows a significant difference between the mean colour intensity of the cytoplasm fixed with 95% ethanol, distilled white vinegar, and apple cider vinegar ($F(2, 6) = 5.23$, $p = 0.049$). Dunnet t-test (post-hoc analysis) was done and suggested that the mean colour intensity of the cytoplasm fixed with apple cider vinegar (M = 104.24, SD = 11.95) was significantly higher than the mean colour intensity of the cytoplasm fixed with 95% ethanol (M = 80.43, SD = 1.72). However, there was no significant difference between the mean colour intensity of the cytoplasm fixed with distilled white vinegar and 95% ethanol.

Evaluation by observers

The buccal cells fixed with each fixative were graded by calculating the total score given by ten observers based on five features which were nucleus details, cytoplasm details, overall morphology, clarity of staining, and uniformity of staining. The total percentage of the overall grade given by the observers to the slides fixed with 95% ethanol, distilled white vinegar, and apple cider vinegar was calculated using the formula, Total percentage = (n/30) x 100%, and the result showed in Fig. 1. The Fleiss' Kappa test was performed to determine the strength of agreement against the overall grade of slides fixed with different fixatives given by 10 observers (raters) as shown in Table III. The strength of agreement for the overall grade of slides fixed with 95% ethanol was determined to be fair, meanwhile, the strength of agreement for the overall grade of slides fixed with distilled white vinegar and apple cider vinegar was determined to be moderate and poor respectively.

Comparison between vinegar-fixed buccal cells and 95% ethanol-fixed buccal cells based on various features

Buccal cells fixed with distilled white vinegar and apple cider vinegar were compared with those fixed with 95% ethanol based on their nucleus size, cytoplasm size, N/C ratio, nucleus colour intensity, and cytoplasm colour intensity. These features were measured quantitatively by using image analysis software which is Toupview

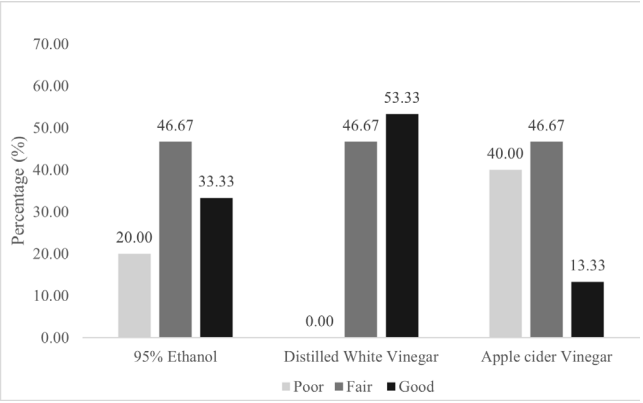

Fig. 1: Comparison of the total percentage of the overall grade assigned by the observers to the slide fixed with 95% ethanol, distilled white vinegar, and apple cider vinegar. The majority of the observers rated the slides fixed with 95% ethanol as "Fair" (46.67%). Slides fixed with distilled white vinegar were predominantly rated as "Good" (53.33%), and slides fixed with apple cider vinegar also received a majority rating of "Fair" (46.67%).

Table II: Colour intensity of buccal cells fixed with different fixatives

Types of Fixative	N1		N2		N3	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
95% Ethanol	145.99	78.58	179.71	81.98	130.69	80.72
Distilled White Vinegar	133.37	86.37	101.51	68.68	142.26	92.55
Apple Cider Vinegar	182.32	90.63	183.96	113.04	198.75	109.05

The unit is in pixels

and then analysed using the independent t-test. The independent t-test (Table IV) showed that the mean difference of nucleus size (t (4) = 0.017, p = 0.987), cytoplasm size (t (4) = 0.368, p = 0.731), N/C ratio (t (4) = -0.849, p = 0.444), nucleus colour intensity (t (4) = 1.276, p = 0.271), and cytoplasm colour intensity (t (4) = -0.292, p = 0.785) between distilled white vinegar and 95% ethanol was not statistically significant. In addition, the independent t-test (Table V) also showed that the mean difference of cytoplasm size (t (4) = 0.368, p = 0.731) and nucleus colour intensity (t (4) = 1.276, p = 0.271) between apple cider vinegar and 95% ethanol was not statistically significant. In addition, the mean difference of nucleus size (t (4) = 1.276, p = 0.271), N/C ratio (t (4) = 1.276, p = 0.271), and cytoplasm colour intensity (t (4) = 1.276, p = 0.271) between apple cider vinegar and 95% ethanol were statistically significant.

DISCUSSION

Ethanol (CH_3CH_2OH), known as ethyl alcohol, remains the gold standard fixative for cytological evaluations. Its ability to dehydrate cells, preserve morphology, and enhance staining properties has strengthened its role in this field (4). However, exploring alternative fixatives with potential advantages is crucial since ethanol has health risks. Ethanol is flammable, volatile with an unpleasant odour, and has been proven to be carcinogenic based on some studies on animal models (4, 6). According to Singh et al. (2019), vinegar may replace ethanol in cell fixation since acetic acid, a compound in vinegar, can help prevent nucleic acid loss and stabilize the nucleus of cells by DNA precipitation. Acetic acid may have fixative characteristics as ethanol due to its acidity and capacity to denature proteins.

This study chose two types of vinegar, distilled white vinegar, and apple cider vinegar, to substitute 95% ethanol as cell fixatives in the Papanicolaou staining protocol. Even though both types of vinegar contain acetic acid for potential cell fixation, their acidity and additional components such as sugars and other organic compounds might influence fixation outcomes (9, 12). Thus, these two types of vinegar were chosen to investigate their potential variations in retaining the cellular morphology of buccal cells. Furthermore, both types of vinegar were selected as ethanol substitutes since they are typically less expensive and more widely available. This is especially helpful in situations with limited resources where it may be difficult to get the gold standard fixatives which in this scenario, ethanol.

Unfixed cells are not immobilized, leading to alterations in their appearance (13). For example, unfixed exfoliated cells in effusion fluid, are viable and freely floating which causes them to become round and plump with well-preserved cytologic details, larger cytoplasm, enlarged nuclei, and prominent nucleoli. This indicates that without fixation, cells tend to swell, and exhibit increased cytoplasmic volume along with enlarged nuclei. Therefore, the evaluation of nucleus size, cytoplasm size, and nuclear-cytoplasmic (N/C) ratio of

Table III: Inter-observer Reliability as Measured by Fleiss' Kappa

Types of Fixatives	Confidence Interval		P-value	Fleiss' Kappa	Strength of Agreement[a]
	Lower limit	Upper limit			
95% Ethanol	0.079	0.325	0.001	0.202	Fair
Distilled White Vinegar	0.355	0.692	<0.001	0.524	Moderate
Apple Cider Vinegar	-0.147	0.113	0.795	-0.017	Poor

[a]Altman (1991) interpretation

Table IV: Comparison between buccal cells fixed with distilled white vinegar and 95% ethanol based on various features

Variables	Distilled white vinegar (n=3) Mean (SD)	95% ethanol (n=3) Mean (SD)	Mean diff (95% CI)	t-stats[a] (df)	P-value
Nucleus size	9.99 (0.32)	10.00 (0.98)	(-1.64, 1.66)	0.017 (4)	0.987
Cytoplasm size	374.88 (6.79)	390.61 (73.63)	(-102.80, 134.26)	0.368 (4)	0.731
N/C ratio	0.029 (<0.001)	0.026 (0.005)	(-0.01, 0.01)	-0.849 (4)	0.444
Nucleus colour intensity	127.05 (23.04)	152.13 (25.08)	(-29.50, 79.67)	1.276 (4)	0.271
Cytoplasm colour intensity	82.53 (12.39)	80.43 (1.72)	(-22.16, 17.94)	-0.292 (4)	0.785

[a] Independent t-test; $p < 0.05$ considered as significantly different

Table V: Comparison between buccal cells fixed with apple cider vinegar and 95% ethanol based on various features

Variables	Apple cider vinegar (n=3) Mean (SD)	95% ethanol (n=3) Mean (SD)	Mean diff (95% CI)	t-stats[a] (df)	P-value
Nucleus size	12.57 (1.18)	10.00 (0.98)	(-5.02, -1.19)	-2.911 (4)	0.044
Cytoplasm size	321.99(8.70)	390.61 (73.63)	(-50.24, 187.47)	1.603 (4)	0.184
N/C ratio	0.042 (0.006)	0.026 (0.005)	(-0.028, -0.003)	-3.465 (4)	0.026
Nucleus colour intensity	188.34 (9.05)	152.13 (25.08)	(-78.95, 6.53)	-2.352 (4)	0.078
Cytoplasm colour intensity	104.24 (11.95)	80.43 (1.72)	(-43.17, -4.45)	-3.415 (4)	0.027

[a] Independent t-test; $p < 0.05$ considered as significantly different

buccal cells after fixation with different fixatives was done. The fixatives used were distilled white vinegar, apple cider vinegar, 95% ethanol as the positive control, and unfixed cells as the negative control. The cells were observed, and the measurement was taken at different time intervals which were after 1 hour, 7 hours, and 24 hours.

Based on Table I, the findings revealed comparable nucleus size in buccal cells fixed with distilled white vinegar and 95% ethanol across the time intervals. In contrast, apple cider vinegar-fixed buccal cells had significantly larger nuclei, resembling unfixed cells. After being fixed with distilled white vinegar, the cytoplasm size of the buccal cells was still comparable to those fixed with 95% ethanol meanwhile the cytoplasm size of buccal cells fixed with apple cider vinegar was significantly smaller compared to 95% ethanol. In addition, the N/C ratio of buccal cells fixed with distilled white vinegar, 95% ethanol, and unfixed cells were comparable meanwhile apple cider vinegar-fixed buccal cells had a higher N/C ratio. Unfixed cells had a comparable N/C ratio because the nucleus and cytoplasm are enlarged uniformly across the time intervals which makes no significant changes in the N/C ratio. On the other hand, apple cider vinegar-fixed buccal cells had enlarged nuclei and shrunk cytoplasm resulting in a higher N/C ratio. Based on the statistical analysis (One-way ANOVA), there were significant differences in the nucleus size at 1 hour ($p = 0.006$) and 7 hours ($p = 0.004$) after fixation where the nucleus fixed with apple cider vinegar was significantly larger than those fixed with 95% ethanol. For the cytoplasm size, there were significant differences in the cytoplasm size at 1 hour ($p = 0.005$), 7 hours ($p = 0.006$), and 24 hours ($p = 0.027$) after fixation but the Dunnett t-test (post-hoc comparison) showed that there was no significance difference in the cytoplasm size of buccal cells fixed with distilled white vinegar and apple cider vinegar compared to those fixed with 95% ethanol. In addition, there were significant differences in the N/C ratio at 1 hour ($p = 0.010$) and 7 hours ($p < 0.001$) after fixation where the N/C ratio of buccal cells fixed with apple cider vinegar was significantly higher compared to those fixed with 95% ethanol. The results corresponded to the findings of a study by Lesmana et al. (2022), which suggested that apple cider vinegar may have cytotoxic effects on cells. The study found that doses of 1.25%, 2.5%, and 5% of the vinegar may be harmful to cells by lowering cell viability to less than 70%. The flavonoid, acetic acid content, and other compounds in apple cider vinegar may contribute to its influence on the nucleus and cytoplasm (15). Overall, buccal cells fixed with distilled white vinegar and those fixed with 95% ethanol are quantitatively comparable in terms of nucleus size, cytoplasm size, and N/C ratio. Nevertheless, buccal cells fixed with apple cider vinegar are quantitatively incomparable.

Next, the colour intensities of the nucleus and cytoplasm of buccal cells fixed with distilled white vinegar, apple cider vinegar, and 95% ethanol (positive control) were measured using Toupview software. The purpose of the analysis was to compare the colour intensities of the nucleus and cytoplasm of buccal cells fixed with different fixatives. Computer-based measurement tool in the software was used to measure the colour intensity by outlining the nucleus and cytoplasm using a digitalized cursor (11). The mean colour intensity was calculated automatically by the software and the procedure was replicated three times using cells from different samples which were fixed with the same fixatives.

Statistical analysis (One-way ANOVA test) was conducted, and the result showed a significant difference in the mean colour intensity of the nucleus fixed with distilled white vinegar, apple cider vinegar, and 95% ethanol since the p-value is less than 0.05. Based on the post-hoc comparison, the mean colour intensity of the nucleus fixed with distilled white vinegar was significantly lower than that fixed with apple cider vinegar, whereas there is no significant difference between the mean colour intensity of the nucleus fixed with distilled white vinegar and apple cider vinegar compared to 95% ethanol (ANOVA, $p < 0.05$). Additionally, as the p-value is less than 0.05, the One-way ANOVA also demonstrated a significant difference in the cytoplasm's mean colour intensity. In particular, the mean colour intensity of the cytoplasm fixed with apple cider vinegar was significantly higher than buccal cells fixed with 95% ethanol, according to the Dunnett t-test (post-hoc analysis). On the other hand, there is no significant difference when comparing the mean colour intensity of the cytoplasm fixed with 95% ethanol with those fixed with distilled white vinegar. The finding is consistent with a study by Koomen et al. (2020) where the study demonstrated that cell lines fixed in different solutions exhibited different staining intensities when subjected to standardized assays. Overall, the colour intensities of the nucleus fixed with distilled white vinegar and apple cider vinegar are comparable to those fixed with 95% ethanol. In addition, the cytoplasm's colour intensity of buccal cells fixed with distilled white vinegar is also comparable to those fixed with 95% ethanol whereas the apple cider vinegar-fixed buccal cells have incomparable cytoplasm's colour intensity.

Furthermore, a qualitative assessment was done based on the evaluation by observers. The slide evaluation was conducted based on the scoring system of five features which were nucleus details, cytoplasm details, overall morphology, clarity of staining, and uniformity of staining with a scoring value of 0 for poor, 1 for fair, and 2 for good. The total score was calculated to give the slides evaluated overall grade which was graded as good (total score ≥ 8), fair (total score 5 to 7), and poor (total score ≤ 4). The observers were selected from the Medical Laboratory Technology (MLT) Department

of UiTM Puncak Alam which were two lecturers with expertise in cytology, one cytology laboratory staff, and seven final-year students who had taken cytology courses and gone for internships in the previous semesters. This evaluation method was implemented based on previous studies with a slight modification and optimization.

Based on the findings, the majority of the observers rated the slides of buccal cells fixed with 95% ethanol as fair (46.67%), those fixed with distilled white vinegar as good (53.33%), and those fixed with apple cider vinegar as fair (46.67%). Fleiss' Kappa was conducted to determine the strength of agreement between the observers (Table II). For the slides fixed with 95% ethanol, the strength of agreement was fair with a Fleiss' Kappa value of 0.202. Next, the strength of agreement for slides fixed with distilled white vinegar was moderate since the Fleiss' Kappa value was 0.524 meanwhile the strength of agreement between observers who rated the slides fixed with apple cider vinegar was poor as the Fleiss' Kappa value was -0.017. A fair Fleiss' Kappa agreement indicates reasonable consistency between the observers in evaluating the slides but there is still room for improvement. Moderate agreement suggests substantial agreement among the observers, while poor agreement indicates significant inconsistency in evaluating the slides by the observers. Low levels of interrater reliability, for instance, may not be acceptable in the healthcare and clinical research fields since they may result in unreliable results if study findings influence clinical practice (17). The findings might show poor agreement for the grade assigned to the slides fixed with apple cider vinegar since there was a limitation to the assessment where the students were not experts in evaluating slides unlike the lecturers and the cytology laboratory staff who had more experience observing slides. Overall, slides fixed with distilled white vinegar and apple cider vinegar were mostly graded as good and fair, respectively, which is qualitatively comparable to slides fixed with 95% ethanol, predominantly graded as fair.

Based on the comparison between vinegar-fixed buccal cells and 95% ethanol-fixed buccal cells based on various features (Table III and Table IV), the findings show that buccal cells fixed with distilled white vinegar have comparable nucleus size, cytoplasm size, N/C ratio, nucleus colour intensity, and cytoplasm colour intensity compared to 95% ethanol-fixed buccal cells since the independent t-test conducted indicated no significant difference between the mean value of each feature (Independent t-tests, $p > 0.005$). On the other hand, apple cider vinegar-fixed buccal cells have comparable cytoplasm size and nucleus colour intensity (Independent t-tests, $p > 0.005$) but incomparable nucleus size, N/C ratio, and cytoplasm colour intensity (Independent t-tests, $p < 0.05$) where the value of each feature was significantly higher compared to those fixed with 95% ethanol. Thus, buccal cells fixed with distilled white vinegar are comparable to 95% ethanol-fixed buccal cells. In contrast, apple cider vinegar-fixed buccal cells are incomparable to those fixed with 95% ethanol.

CONCLUSION

The study concluded that distilled white vinegar can effectively become an alternative to 95% ethanol as a fixative for buccal cells, supported by the comparable result between buccal cells fixed with distilled white vinegar and those fixed with 95% ethanol from the qualitative and quantitative assessments. In contrast, apple cider vinegar is not an effective cell fixative compared to 95% ethanol. This is because some features of the buccal cells fixed with apple cider vinegar were significantly different compared to those fixed with 95% ethanol.

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