ORIGINAL ARTICLE

Leaves aqueous extract as a cytological stain for buccal cell screening

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Abstract:

This study describes the preparation and use of *Alternanthera dentata* (purple knight), *Alternanthera ficoidea* (grenadine), *Brassica oleracea* (red cabbage), *Amaranthus dubius* (red spinach) and *Lawsonia inermis* (henna) for the differential staining of buccal cells to find out its staining effect on nuclear and cytoplasm as an alternative to Papanicolaou stain in cytology laboratory. The leaves aqueous extracts were tested with or without addition of Aluminium chloride ($AlCl_3$) mordant using regressive Papanicolaou staining procedure as a standard with minor modification. All the leaves extract was measured for pH, physical colour concentration, qualitative and quantitative staining intensity and ability. Statistical data were calculated by using Weighted-Kappa analysis showed a fair correlation between staining intensity of leaves aqueous extract dye with Papanicolaou stain while, One-way ANOVA showed no significant difference between leaves aqueous extract and Papanicolaou stain. Therefore, the leaves aqueous extracts stain were not comparable to the standard. As a conclusion, the leaves aqueous extract used in this study has the ability to stain both nucleus and cytoplasm by its own colour; however, the colour intensity is incomparable with Papanicolaou stain as the standard.

Keywords: *Alternanthera dentata* (purple knight), *Alternanthera ficoidea* (grenadine), *Amaranthus dubius* (red spinach), *Brassica oleracea* (red cabbage), *Lawsonia inermis* (henna) Papanicolaou stain

1. INTRODUCTION

Cytology is a study of normal and malignant cell morphologies under light microscope. Cell morphology differentiation is enhanced by using Papanicolaou stains. The stain provide contrast to the cell structures as well as highlighting the features of interest. Stains are classified by their nature, which are synthetic and natural. The natural stain obtained from natural resource such as plants, insects, animals, clays, and minerals are less expensive compare to the chemical mixture synthetic stain [1]. Recently, there has been postulated that the source of natural stain were depleting. Hematoxylin obtained from *Haematoxylum campechianum* (logwood tree) was inadequate due to the shortage which occured in the late 1920s and early 1970s followed in 2008. Hematoxylin shortage occurs due to several disruptions such as during World Wars I and II [2]. In addition, hematoxylin is expensive due to transportation cost since it is imported from Caribbean island, Indian and Pacific Oceans island [2].

Other than hematoxylin, Eosin Azure and Orange G components are a part of Pap stain, derived from the synthetic stain. Despite of having superior stronghold properties in staining process, these two types of synthetic stain are offering a convenient usage with a good stability, various choices of colours, and most importantly, cost saving [3]. The synthetic dyes are very efficient, but the dyes are hazardous to both human and animal health. There are risk of cytotoxic and carcinogenic to mammalian cells, which may cause a liver tumor, reduce the capacity of food intake, skin allergies, decrease growth and fertility rates [1, 4]. In addition, it was also reported as pollution problems once being released to the environment due to their non-biodegradable properties and also reduce soil fertility [5]. Therefore, this study was carried out to investigate the use of natural stain extract as an alternative to compliment this problem.

2. MATERIALS AND METHODS

2.1 Leaves collection and extraction

Five different types of leaves were collected locally. The plants were washed with distilled water and dried in an oven at 40^{o}C for 48 hours [6]. Dried leaves were finely grounded using homogenizer. One hundred mL of distilled water was added to each 10 grams of ground leaves. The powdered leaves were mixed with distilled water in an Erlenmeyer

flask and then incubated at 60°C for 1 hour in the shaker incubator. Leaves aqueous extract solution was filtered through gauze, and filtered again using Whatman's filter paper and then placed in a Erlenmeyer flask and protected from light by sealing the flask with aluminium foil until further use. The solution was stored at 4°C and stable for 2 months [7].

2.2 Buccal smear collection

The experiment was approved and endorsed by the UiTM Human Ethics Committee (600-IRMI (5/1/16). Five healthy students volunteered for the collection of samples from buccal mucosa. Students rinsed their mouth using the tap water twice followed by 0.9% normal saline. Buccal surface of the cheeks was scraped using wooden spatula, smeared on glass slides and fixed into the Coplin-jar containing 95% ethyl alcohol for 30 minutes [8].

2.3 Preparation of leaf aqueous extract with mordant

Mordant preparation used a 1:1 ratio, by admixed 5 ml of leaf aqueous extract and mark up to 10 ml with aluminium chloride [9].

2.4 pH and colour concentration measurement

Leaves aqueous extract with and without mordant pH was measured using pH meter (Knick, Germany). Staining colour concentration was measured with optical density (OD) value using UV-Visible absorbance spectrophotometer at wavelength 569 nm (hematoxylin) and 528 nm (eosin) [10, 11].

2.5 Staining process

Regressive Papanicolaou stain technique was used to compare three group slides comprised of control, mordant and without mordant. The procedure was used standard protocol of Pap stain by Leica, US. Slides were then mounted with DPX mounting. Slide were observed using camera mounted light microscope (Leica ICC50 HD, Germany) at x10 and x40 magnification. Observation was recorded in the following form.

Observed by:	Date:	Plants used:			
Time:	Slide Number:	Stains to be substituted:			
Cell	Structure	Staining Ability (Tick ✓)			
		Poor	Fair	Good	Very good
Superficial	Nucleus/ cytoplasm				
Intermediate	Nucleus/ cytoplasm				

Notes:

- Poor- refer to the absence of stain on the nucleus/ cytoplasm and no nuclear details / difficult appreciation on cytoplasm can be seen.
- Fair - refers to the presence a trace of stain on the nucleus/ cytoplasm and no nuclear details/ difficult appreciation on cytoplasm can be seen.
- Good - refers to the presence of stain on the nucleus/ cytoplasm comparable to hematoxylin dye. Presence of nuclear details but chromatin not clearly defined/ difficult appreciation on cytoplasm.
- Very good - refers to the presence of stain on the nucleus comparable to hematoxylin dye. Presence of nuclear details clearly defined

2.6 Qualitative and quantitative analysis

For qualitative data analysis, ten Medical Laboratory Technology (MLT) staff and students was selected to observe the stained slides and results were recorded in the observation form. The observation form was obtained from the previous study [12]. The observation form was divided into two, which are for nucleus stain and cytoplasm stain and software IBM Statistical Package for Social Sciences (SPSS) version 23 was used for Weighted-Kappa and One-way ANOVA test. Statistical analysis was done by calculating P value. Weighted kappa is a broadly utilized measurement for abridging between rater agreements on a categorical data [13].

Quantitative analysis was measured using MIPAR (Materials Image Processing and Automated Reconstruction) Image Analysis Software. MIPAR is a progressive picture investigation programming, fit for recognizing and measuring highlights from about any caught picture from an electron or light microscope [14].

3. RESULTS

Detail of the results was presented in Table 1. *A. dentata* (purple knight) extract with $AlCl_3$ mordant stained the nucleus of superficial cells with pink colour while pale pink was stained without mordant. Meanwhile, intermediate cell nucleus stained with mordant gave purple colour but pale purple without mordant.

A. ficoidea (grenadine), *B. oleracea* (red cabbage) and *A. dubius* (red spinach) extract with mordant, stained superficial cell nucleus as pale pink and pale purple for the nucleus intermediate cell. The following extract without mordant stained both superficial cell and intermediate cell nucleus pink and purple colour respectively. *L. inermis* (henna) extract with aluminium chloride ($AlCl_3$) mordant stained superficial cell cytoplasm as blue colour, while without addition of mordant showed light brown staining. Control slide stained with Papanicolaou stain showed dark blue nucleus and eosinophilic colour cytoplasm in both superficial and intermediate cell.

Table 1: Overview of the results.

Type of leaf extract	Type of dye	pH	Physical colour	Colour concentration (%)	Nuclear stain		Cytoplasm stain		Colour intensity		
					Superficial	Intermediate	Superficial	Intermediate	**Kappa agreement**	**Mipar (pixel)** superficial cell	**Mipar (pixel)** intermediate cell
Alternanthera dentata (purple knight)	H	2.23	Dark purple	100.00	Dark purple	Dark purple	Pink	Blue	ND	159.0481	142.2475
	M	1.94	Slightly dark red	54.39	Pink	Light purple	ND	ND	Fair	105.5134	98.5988
	WM	4.00	Red	80.41	Pink	Light purple	ND	ND	Poor	105.5157	89.1546
Alternanthera ficoidea (grenadine)	H	2.23	Dark purple	100.00	Dark purple	Dark purple	Pink	Blue	ND	177.6916	160.9981
	M	1.91	Slightly dark red	68.37	Pink	Light purple	ND	ND	Poor	113.0489	78.0575
	WM	5.47	Red	98.42	Pink	Light purple	ND	ND	Poor	103.8423	88.6392
Brassica oleracea (red cabbage)	H	2.23	Dark purple	100.00	Dark purple	Dark purple	Pink	Blue	ND	179.0907	127.8884
	M	1.68	Dark purple	99.62	Pink	Light purple	ND	ND	Fair	97.6410	84.0956
	WM	4.24	Red	66.48	Pink	Light purple	ND	ND	Fair	117.7663	92.8138
Amaranthus dubius (red spinach)	H	2.23	Dark purple	100.00	Dark purple	Dark purple	Pink	Blue	ND	170.0813	177.4302
	M	1.86	Dark brown	49.84	Pink	Light purple	ND	ND	Fair	111.0794	78.9587
	WM	6.25	Light brown	60.35	Pink	Purple	ND	ND	Fair	139.8489	120.7699
Lawsonia inermis (henna)	EA50	5.24	Dark pink	100.00	Dark purple	Dark purple	ND	ND	ND	94.2011	81.3305
	M	2.00	Brown	51.81	ND	ND	Light blue	Light brown	Fair	64.7900	7.1322
	WM	4.36	Brown	58.48	ND	ND	Light blue	Light brown	Fair	59.8859	61.5845

H: hematoxylin; M: mordant; WM: without mordant; EA50: eosin azure 50; ND: not done

4. DISCUSSION

All selected leaves were based on the physical appearance, availability in Malaysia and literature review of previous study. For the nuclear stain, deep purple leaves from *Alternanthera dentata* (purple knight), *Alternanthera ficoidea* (grenadine), *Brassica oleracea* (red cabbage) and *Amaranthus dubius* (red spinach) were selected while *Lawsonia inermis* (henna) was used for cytoplasm stain.

Although huge numbers of natural stain initially have poor affinity for the tissues and some limitation to yield colour, this shall be solved with an aid of some chemical mixture adjustments with the metal salt formation, commonly refer as a mordant [15]. Mordant is commonly included in the staining procedure in order to fix or increase the staining intensity [10]. The combination of mordant with natural dyes may enhance the colour or may give a different colour [10]. Upon the addition of mordant, most of the physical leaves aqueous extract showed changes in their colour. The addition of $AlCl_3$ mordant changed the original physical colour of *A. dentata* and *A. ficoidea* extract from red to slightly dark red. *B. oleracea* extract changed from red to dark purple, while *A. dubius* extract changed from light brown to dark brown. Remarkably, *L. inermis* showed no changes of colour with the addition of mordant. However, the physical stain of all extracts were incomparable neither to Hematoxylin nor Eosin stain. In this study, stain to mordant ratio has been optimised to 1:1 but the staining intensity does not increase.

pH has a direct impact on affinity and ability of stain to bind to the cells. Leaves aqueous extract pH was acidic within the range of 4.09 to 6.28. Upon the addition of mordant, the pH dropped in-between 1.69 and 1.95 as it has become more acidic. Therefore, high concentration of hydrogen produced a positive charged of protein, which then enables the protein to bind with the negative charges of dyes [15]. In this case, too acidic pH with high hydrogen concentration appeared to ruin the stain's affinity for cell nuclei and other cell's structures. Acidic structures in nucleus should be stained with basic dyes (hematoxylin) while basic structures of cytoplasm should be stained with acidic dyes (eosin) [10].

Colour intensity was further evaluated by qualitative human observation and software quantitative measurement. The appointed panels were required to screen the slide and determine if the cells can pick up the extracted aqueous dye to give out colour. The stained slides were examined using the light microscope and graded as poor, fair, good, or very good. In hospital, human observation was a routine practice to observe the slide to check whether the cell is normal or abnormal.

The extract with mordant for *A. dentata* gave better staining quality to the nucleus of superficial and intermediate cell with the pink and purple colour, respectively. Staining without mordant on *A. ficoidea*, *B. oleracea* and *A. dubius* has better staining quality compared with addition of mordant. On the other hand, *L. inermis* with mordant gave better staining outcome compared with the absent of mordant. MIPAR (Materials Image Processing and Automated Reconstruction) image analysis software was used to conduct quantitative examination to double check the result that has been read by the human observer [14]. The cell images were captured and uploaded to measure the intensity. Slide observation by human naked eyes have a risk of bias that needed to be avoided. Human bias refers to the tendency of human to seek out, attend to, and sometimes embellish experiences which support or confirm their beliefs [16]. Therefore, this software reduced human bias and value of staining intensity measurement can strengthen the qualitative analysis.

Both quantitative and qualitative results of the staining intensity was related. Results were strongly agreeable that alternative dyes may stain the cell, but it is not comparable with Papanicolaou stain. Statistical analysis of weighted kappa revealed a fair correlation between staining intensity of leaves aqueous extract dye with Papanicolaou stain, therefore the dyes are not comparable to the gold standard. The result indicates low possibility of using this leaves as an alternative even though it can still be used because there is an uptake in the cell but does not support by the mordant.

It was challenging to standardize the staining results, which utilizes the natural product as dyes. Though some plants may possess similar character, the staining outcome may differ due to other factors such as atmosphere, soil, growth period and cultivation techniques. Furthermore, almost all natural dyes require a mordant for fixing or to increase the intensity of the colour on the stained tissues. Generally, metallic mordant such as alum and iron are used and applied in the dye solutions but may cause health and disposal hazard. Therefore, natural mordant could be an option to substitute the metal mordant for safer tissue staining. Many other factors should be evaluated if the natural dyes are used for tissue or cell staining. Firstly, the spring of the selected plant, should be accessible in the region. It must be easily available to decrease the cost and to avoid raw material deficiency.

5. CONCLUSION

The results of *A. dentata, A. ficoidea, B. oleracea, A. dubius and L. inermis* aqueous extracts used in this study has demonstrated their staining potential specifically towards the cell nucleus and the cytoplasm but, color intensity produced was not comparable with Papanicolaou stain which is the gold standard.

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