

THE EFFICACY OF SANTAN FLOWER (*Ixora occinea Linn.*) AS AN ALTERNATIVE STAIN TO EOSIN Y IN WRIGHT-GIEMSA STAIN

Patricia Erika C. Cruz, Almira P. De Vera, and Anne Denielle M. Villa

ABSTRACT

*Despite the advances in hematology automation and application of molecular techniques, the peripheral blood smearing has remained a very important diagnostic test to the hematologists. Though Eosin Y is widely used for staining in peripheral blood smearing, it is necessary to develop an alternate potential stain to replace it due to its hazardous nature. A stain should be eco-friendly, safe with good visual clarity, and staining characteristics. This study aimed to develop the extract of *Ixora coccinea Linn.* (santan flower) as an alternative stain to eosin because its proven cyanidin, flavonoids, and most importantly, anthocyanin content make a good natural colorant source. The flower extract was prepared with the solvent ethanol. Following the procedure of wright giemsa stain in preparing peripheral blood smear, the extract of santan was used as an exchange for commercially prepared Eosin y stain. The characteristics of blood cells in the smear and the intensity of the color were examined under the microscope. Ten percent (10%) concentration, 25%, and 36% were used to assess the parameters like degree of transparency, visual clarity, and uniform of staining. After experimentation and gathering all the results, it was concluded that *Ixora coccinea Linn.* (Santan) extract is still not an effective alternative stain for WBCs on the PBS. It poorly differentiated the types of white blood cells and barely demonstrated cell morphologies strongly indicating poor effectivity of the solution therefore decreasing the quality of stained smear and causing difficulties in differential counts.*

Keywords: Peripheral blood smear, Eosin y, Wright-Giemsa stain, *Ixora coccinea Linn.*

INTRODUCTION

Peripheral blood film (PBF) is a laboratory work-up that involves cytology of peripheral blood cells smeared on a slide. It is invaluable in the characterization of various clinical diseases. Furthermore, it is a highly informative haematological tool at the clinician's disposal in screening, diagnosis and monitoring of disease progression and therapeutic response. It commonly utilizes Romanowsky stains, mixtures of acidic dye and basic dyes that gives differential staining of the different cellular components. These consist of Eosin Y or Eosin B with methylene blue and/or any of its oxidations products. There are a number of special stains employed to identify specific inflammatory cells seen in peripheral blood and tissues (Keohane, Smith & Walenga, 2016).

According to Adewoyin and Nwogoh (2014), commonly used stains are Wright-Giemsa stain and Leishman stain which both contains methylene blue which is basic, and eosin, an aniline acidic dye. This proves that Wright-Giemsa stain is a polychrome stains because they contain both eosin and methylene blue as stated by Keohane, Smith and Walenga (2016).

Moreover, others state that not only does eosin act as stain; it also serves as a fixative. Eosin is also a part of Hematoxylin and eosin or H&E stain (Fischer & Cardiff, 2006). Despite all its uses and advantages in the field, Eosin Y was considered hazardous as stated by the Occupational Safety and Health Act 29 CFR 1910. It may incur further disability to those with impaired respiratory function, airway diseases and conditions such as emphysema. It may also produce systemic injury with harmful effects if allowed access to open cuts, abraded or irritated skin. In addition, it is also a skin, eye and mucous irritant which may result to chelitis, stomatitis and dermatitis. Finally, indefinite reports state that Eosin Y is an animal carcinogen which means that it can both be considered as a health and at the same time environmental hazard (US Government Publishing Office, 2013).

The researchers desire to find an alternative solution for eosin considering safety and cost effectiveness as the theme of today's studies. Itodo et al. (2014) proved that *Allium cepa* (red onion), *Brassica oleracea* var. *capitata f. rubra* (red cabbage), Hibiscus, and rose are some of the effective alternatives.

Accordingly, the goal of this study is to find an alternative to Eosin Y, due to the harm and disadvantages that it may cause and develop the extract of *Ixora coccinea* Linn. (Santan flower) into an alternative staining solution to eosin in the peripheral blood smear because of its proven cyanidin, flavonoids, and most importantly, anthocyanin content which makes it a good natural colorant source (Medical Health Guide, 2011).

Objectives of the Study

This study aimed to ascertain the efficacy of *Ixora coccinea* Linn. as an alternative stain for Eosin Y on staining white blood cells on the peripheral blood smear. More specifically, it aimed to: (1) determine if there is a significant relationship between the control, Wright-Giemsa stain and the two concentrations, 25% and 36%, in the peripheral blood smears in terms of: (a) degree of transparency, (b) visual clarity, (c) cell morphology demonstration and (d) uniformity of staining; (2) determine if there is a significant difference between the santan extract, 25% and 36% concentrations with the control, Wright-Giemsa stain; (3) compare the stability of the santan extracts, 25% and 36% and the control, Wright-Giemsa stain by monitoring their daily appearance and artifact formation in the span of 7 days and; (4) produce a cost efficient alternative stain to Eosin Y in Wright-Giemsa stain from santan flower (*Ixora coccinea* Linn.).

METHODOLOGY

In this study, true experimental design was applied, a design that has control group and a complete control over extraneous variables.

Santan flowers (*Ixora coccinea* L.) utilized in this study were obtained from Sta. Rosa, Laguna on an afternoon with fair weather. The flowers were placed in a clean plastic container with sealed lid and stored in room temperature for 24-48 hours before being processed. While in obtaining blood samples from 30 students of LPU-Laguna, needle and syringe method of venipuncture were used.

Extraction and preparation of the solution were done in line with the study of Chukwu (2011) and Adegoke et al. (2010) where the plant material (santan flower) was washed and dried under the shade for

minimum of 7 days and homogenized to a fine powder using mortar and pestle, packaged in glass jars and was stored in a refrigerator at 4°C until required for use. Furthermore, the powdered plant material was soaked in absolute ethanol for 3-4 days under room temperature and filtered by using Whatman No 1 filter paper. The extract was transferred to a sterile container and stored in a 4°C until use.

Preliminary testing was done using the initial plant extract of 10% concentration to stain 15 blood samples. For the second trial, two (2) concentrations were prepared with 33.85g of santan powder mix with 101.5mL of ethanol making up the 25% concentration and a total volume of 134.4 g/mL and the 36% concentration, comprised of 60.18mL ethanol and 33.85g santan powder, a total volume of 94.03g/ml. These different concentrations were used to assess the concentration that would produce effective stain for WBCs

Staining was done by placing 0.05 mL (50 uL) of blood on a slide and spread lightly to rapidly produce a smear. The slide was fixed in the methanol for 5 minutes and dried. Methylene blue was placed upon the smear for 2 minutes and then rinsed with running water. Eosin Y was added on the smear for 1 minute. Then, the slide was rinsed again with water and let to dry (Abnova, 2010). All steps were repeated in another slide except that the santan flower extract was used in exchange of Eosin Y and for each blood sample, three smears were made sorted according to the following groups: Group I (Control Group): Stained with Wright-Giemsa stain, Group II: Stained with 25% santan extract, and Group III: Stained with 36% santan extract.

The stained smears were graded as: (1) *poor* (not clear and no details with cells barely seen); (2) *good* (clear and detailed having defined cell structures but poorly demonstrated morphology) and (3) *excellent* (very clear, fine and very detailed with all the necessary identification structures seen and well differentiated cells per field).

RESULTS AND DISCUSSION

Using the alternative santan solution of both 25% and 36% concentrations, results showed that santan extract gives poorly stained smears graded as (1) one compared to that of the commercially Eosin Y reagent as shown on the table below:

Criteria	Types of Cells	25% Santan Extract																															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
Degree of Transparency	Neutrophils	2	2	1	2	1	1	2	2	2	1	1	2	1	2	1	1	1	1	1	2	1	1	2	2	2	1	1	1	1	1		
	Lymphocytes	2	2	1	2	1	1	2	2	2	1	1	2	1	2	1	1	1	1	1	1	2	1	1	2	2	2	1	1	1	1		
	Monocytes	2	2	1	2	1	1	2	2	2	1	1	2	1	2	1	1	1	1	1	2	1	1	2	2	2	1	1	1	1	1		
	Eosinophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0		
	Basophils	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0		
Visual Clarity	Neutrophils	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1
	Lymphocytes	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	
	Monocytes	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Eosinophils	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	
	Basophils	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	
Demonstration of Cell Morphology	Neutrophils	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Lymphocytes	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Monocytes	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Eosinophils	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Basophils	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	
Uniform Staining	Neutrophils	3	3	3	2	3	3	2	3	3	3	3	2	2	3	3	2	1	2	1	2	2	1	2	1	1	2	2	2	1	1	1	
	Lymphocytes	3	3	3	3	3	2	3	1	3	2	2	1	3	2	2	1	1	2	1	2	1	1	1	2	1	2	2	1	2	1	2	1
	Monocytes	3	3	3	3	3	2	2	3	2	2	1	2	2	2	1	1	1	2	1	2	2	2	1	1	2	1	2	2	1	2	2	1
	Eosinophils	0	0	0	0	0	0	0	0	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	
	Basophils	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0		

Types of Cells	36% Santan Extract																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30			
Neutrophils	2	2	2	2	1	2	2	1	1	1	2	1	1	2	1	2	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1		
	Lymphocytes	2	2	2	2	1	2	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1		
	Monocytes	2	2	2	1	1	2	1	1	1	1	1	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1		
	Eosinophils	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	Basophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
Lymphocytes	2	2	2	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Monocytes	2	2	2	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1
	Eosinophils	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Basophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Neutrophils	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Monocytes	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Eosinophils	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Basophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Neutrophils	3	2	3	2	1	2	3	2	3	1	2	2	2	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Lymphocytes	3	2	3	2	1	2	3	2	3	1	2	2	2	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Eosinophils	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Monocytes	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Basophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Neutrophils	3	2	3	2	1	2	3	2	3	1	2	2	2	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	Lymphocytes	3	2	3	2	1	2	3	2	3	1	2	2	2	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	

The 25% extract showed better relationship having moderately low negative to perfect positive relationship with the control while the 36% concentration extract has very low negative to moderately low positive relationship with the control Eosin Y in terms of the degree of transparency, visual clarity, cell morphology demonstration and

uniformity of staining shown on the tables below:

Table 1. Relationship of 25% santan extract versus 36% Santan extract with the control: Wright Giemsa stain in terms of the degree of transparency in peripheral blood smear staining

Types of Cells	25% Santan Extract			36% Santan Extract		
	R	Interpretation	r^2	R	Interpretation	r^2
Neutrophils	0.20	Very low positive	4.07%	0.07	Very Low positive	0.53%
Lymphocytes	0.36	Moderately low positive	12.70%	0.05	Very low positive	0.23%
Monocytes	UD	UD	UD	UD	UD	UD
Eosinophils	0.56	High positive	31.03%	-0.06	Very low negative	0.38%
Basophils	1	Perfect positive	100%	UD	UD	UD

r- Coefficient of Correlation, r^2 -Coefficient of Determination; UD-Undetermined

Table 2 Relationship of 25% santan extract versus 36% santan extract with the control: Wright Giemsa stain in terms of the visual clarity in peripheral blood smear staining

Types of Cells	25% Santan Extract			36% Santan Extract		
	R	Interpretation	r^2	R	Interpretation	r^2
Neutrophils	0.64	High positive	41.56%	0.37	Moderately low positive	13.50%
Lymphocytes	-	Moderately low negative	6.56%	0.15	- Very low negative	2.39%
Monocytes	0.53	High positive	28.57%	0.34	Moderately low positive	11.82%
Eosinophils	1	Perfect positive	100%	-	Very low negative	0.38%
Basophils	1	Perfect positive	100%	UD	UD	UD

r- Coefficient of Correlation, r^2 -Coefficient of Determination; UD-Undetermined

Table 3 Relationship of 25% santan extract versus 36% santan extract with the control: Wright Giemsa stain in terms of cell morphology demonstration in peripheral blood smear staining

Types of Cells	25% Santan Extract			36% Santan Extract		
	R	Interpretation	r^2	R	Interpretation	r^2
Neutrophils	UD	UD	UD	UD	UD	UD
Lymphocytes	UD	UD	UD	UD	UD	UD
Monocytes	UD	UD	UD	UD	UD	UD
Eosinophils	0.63	High positive	39.64%	-0.09	Very low negative	0.79%
Basophils	1	Perfect positive	100%	UD	UD	UD

r- Coefficient of Correlation, r^2 -Coefficient of Determination; UD-Undetermined

Table 4 Relationship of 25% santan extract versus 36% santan extract with the control: Wright Giemsa stain in terms of the uniform staining in peripheral blood smear staining

Types of Cells	25% Santan Extract			36% Santan Extract		
	R	Interpretation	r^2	R	Interpretation	r^2
Neutrophils	0.63	High positive	40.01%	0.44	Moderately low positive	19.26%
Lymphocytes	0.54	High positive	29.70%	0.44	Moderately low positive	19.38%
Monocytes	0.57	High positive	32.85	-	Very low negative	4.02%
Eosinophils	0.98	Very high positive	96.64	UD	UD	UD
Basophils	1	Perfect positive	100%	UD	UD	UD

r- Coefficient of Correlation, r^2 -Coefficient of Determination; UD-Undetermined

Furthermore, with this showed results, 25% concentration solution was evidently more effective than the 36% concentration due to the greater similarity that it showed with the control. This is further shown on the table below:

Table 5 Comparison between the significant difference of 25% and 36% concentration of santan extract and the control: Wright-Giemsa stain

WBC	T-test Results				Significant Difference
	Degree of Transparency		Visual Clarity		
Neutrophils	25% 0	36% 0	25% 0	36% 0	YES
Lymphocytes	0	0	0	0	YES
Monocytes	0	0	0	0	YES
Eosinophils	P=0.1609	P=0.1609	P=0.4266	P=0.1609	NO
Basophils	P=0.5232	P=0.1608	P=0.5232	P=0.1608	NO
WBC	Cell Morphology Demonstration		Uniform Staining		Significant Difference
	25%	36%	25%	36%	
Neutrophils	0	0	P=0.0636	0	YES
Lymphocytes	0	0	P=0.0036	0	YES
Monocytes	0	0	P=0.0013	0	YES
Eosinophils	P=0.4266	P=0.2760	P=0.7239	P=0.0831	NO
Basophils	P=0.5232	P=0.1608	P=1.0000	P=0.1608	NO

Despite this result, the alternative solutions produced were still not ideal in replacing the commercially prepared Eosin Y reagent. This is in line with the study by Eco & Amir (2014) which showed that not all phytochemical containing plants could be good alternative stains due to the different factors that affect them. In addition, Brown (2012) stated that affinity of staining solutions is affected by different factors including the concentration and type of solvent used.

Lastly, the santan extract was proven more cost efficient than the commercially available Eosin Y reagent costing only PHP3.75 per slide as compared to PHP5.55 per smear of the control Wright Giemsa stain elaborated more on the table below:

Table 8 Comparison of the cost per smear of Wright Giemsa stain and santan extract

	Wright Giemsa Stain			Santan Extract		
	(1L)	(1 gtt)	(1 smear)	(1L)	(1 gtt)	(1 smear)
Methanol	250.00	0.25	0.75	250.00	0.25	0.75
Methylene Blue	800.00	0.8	2.4	800.00	0.8	2.4
Eosin y	800.00	0.8	2.4	—	—	—
Ethanol	—	—	—	200.00	0.2	0.6
Total:	1,850.0	1.85	5.55	1,050.0	1.25	3.75
	0		0			

CONCLUSION

Despite the positive relationship that both 25% and 36% concentrations demonstrate, *Ixora coccinea* Linn. (santan) extract is still not an effective alternative stain for WBCs on the Peripheral blood smear due to the varying results obtained, poor differentiation of WBCs, unclear demonstration of cell morphologies and poor quality produced. Thus, an amount of 25% santan extract solution was concluded as more potent in stain for WBCs on the peripheral blood smears than the 36% concentration. Lastly, Both concentrations are stable as long as proper storage and other considerations were observed and both concentrations of santan extract are cheaper forms of staining solution but they are obviously not as effective as the routinely used Eosin Y in Wright-Giemsa stain.

RECOMMENDATIONS

For better extraction process, the researchers suggest utilizing a mechanical compressing machine instead of compressing the santan flowers by hands to ensure total extraction of solution from the soaked flowers, and using another solvent for extracting *Ixora coccinea Linn*, aside from ethanol and utilizing an alternative method of extraction such as Soxhlet extraction to effectively collect sufficient anthocyanin for the extract.

It is also recommended to store the extract in room temperature instead of placing it in the refrigerator with 4° C to possibly minimize the chance of diluting the extract due to the accumulation of moisture in the glass container also affecting the pH level of the solution making it more basic contradicting the ideal acidic pH level.

For improved extract quality, the researchers recommend monitoring the pH of the solution for several days to assure its stability and maintain its acidity. Lastly for improved staining procedure, utilizing a standardized time of staining and increasing the amount concentration of santan with the same recommended percentage of diluents are suggested.

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