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Efficacy of Bougainvillea Spectabilis (Bougainvillea) Bracts Crude Extract As an Alternative to Wright Stain in Blood Smear Preparation

Hannah Joyce R. Calilung[1], Aileen L. Castillo[1],Cyra Alexandria U. Gasmido[1], Chelsey Anne S. Legaspi[1] and Romy M. Castor[1]
[1]Lyceum of the Philippines (LPU), Manila Campus,
Muralla St, Intramuros, Manila, 1002 Metro Manila, Philippines.
*Corresponding author:romy.castor@lpu.edu.ph

ABSTRACT
In this work, the staining efficacy of Bougainvillea crude extract was evaluated by comparing with the Wright stain as alternative in blood smear preparation. This was studied on the blood collected from 15 non-anemic participants of Lyceum of the Philippines University Cavite. Hematocrit determination was made to confirm if the participants are surely non anemic. Staining pattern of the prepared smear were limited to the effects of Bougainvillea crude extract and Bougainvillea crude extract combine with methylene blue as compared to the standard staining technique, the Wright stain. In addition, the efficacy of Bougainvillea crude extract was further graded by two external and two internal registered medical technologies. Results showed that the staining efficacy of Bougainvillea crude extract is not comparable to the result of Wright stain as all the p-values are less than 0.05. Statistically, the staining capacity of Bougainvillea as alternative to Wright stain showed a significant difference implying that this is not effective in staining the blood smear for morphology evaluation of the blood cells in manual CBC.

Keywords
Bougainvillea spectabilis, crude extract, staining, blood smear, Wright stain

1. INTRODUCTION

The Bougainvillea variety is a flower that is available all over the world [1]. It is associated with the Nyctaginaceae family and contains approximately 18 species [2]. Bougainvillea is prevalent to South America and its existence was first discovered in Brazil in 1778, before Louis Antoine de Bougainville, the French army administrator, introduced it to Europe [2]. They are shrubs scattered in the vines or in the small trees. In addition, they have stems with internodes and straight or marginally bent thistles [3]. The leaves are petiolate, curved, or more extensive to the base. The flowers are exhibited in different shades, depending on the species and cultivars, They blossom consistently throughout the year [3].

Bougainvillea spectabilis is a natural plant of South America which has expanded across the humid and hot environments around the globe [4]. Furthermore, the wide versatility of Bougainvillea spectabilis in different agro-climatic conditions and easy replication has made it a well-known and elaborate crop in the world [5]. Moreover, since it is a dry season and a healthy exposure crop, it is ideal for planting in different areas.In addition, it has been reported that Bougainvillea spectabilis has medicinal values and uses such as anticancer, antifertility, antibacterial, antihyperlipidemic, anti-inflammatory, antifungal, antidiabetic, antiviral and antioxidant [6].The therapeutic effects of Bougainvillea spectabilis were attributed to phytoconstituents elements such as flavonoids, oxalates, alkaloids, glycosides and phenolic mixtures, which are believed to be the basis of its beneficial properties [7].

Several studies have been reported in the analysis of the characteristics of Bougainvillea spectabilis for various uses and applications. Carillo et al. [8] analyzed the morphologicalgrowth of Bougainvillea spectabilis with salt water and found that

Bougainvillea is a salt-tolerant plant and a potential option for cultivation purposes.Bharathi et al. [9] investigated the antibacterial properties of Bougainvillea spectabilis by synthesizing it with nano silver molecules and found that the synthesized components exhibited strong antibacterial behaviour. Ghogar and Jiraungkoorskul [10] examined the antifertility characteristics of Bougainvillea spectabilis, and found that Lee found that the physical elements of Bougainvillea spectabilis impeded spermatogenic processes leading to a reduction in sperm count.Baron et al. [11] scrutinized the ability of Bougainvillea spectabilis to absorb lead and found that it was able to absorb the lead released into the air from the lead paint coated surface in the range of 2.4 ppm to 14 ppm.Zhang et al. [12] investigated the implications of lighting on the growth rate of Bougainvillea spectabilis and found that the flowering of Bougainvillea was significantly influenced by the quality of light received.Rauf et al. [13] evaluated the use of Bougainvillea spectabilis extract with zinc oxide nanomaterial as an anticancer component and found that this material combination exhibited effective anti cancer behavior towards the cancer cells.

Staining is used to show the parts and structures, to reveal the chemical nature and to influence the growth and development of the visibility of the microscopic appearance [14]. Over the decades, researchers have been engaged in enhancing and discovering alternatives to the staining process. The evolution of the stains was developed and greatly improved from natural to synthetic [14]. Likewise, studies have also reported that the concentration of bougainvillea bracts has an intriguing potential as dyeing and staining materials [15]. Thus, this study has explored intends to explore the staining efficacy of Bougainvillea spectabilis in blood smear. This study was done to determine the efficacy of the Bougainvillea as an alternative dye to Wright stain.

2. METHODOLOGY

2.1 Research Design

The authors used the experimental group design. The efficacy of Bougainvillea as an alternative to Wright stain was evaluated by identifying blood cells in a non-anemic population group. Intensity of color and cellular morphology of crude Bougainvillea is observed in non-anaemic blood clotting. A comparative study was also made through the differentiation of Bougainvillea crude extract, Bougainvillea crude extract with methylene blue and Wright stain through color of the nucleus and cytoplasm of RBC nucleus, cytoplasm and lobules of WBC and platelets.

2.2 Participants

The study used convenience-sampling technique in selecting non-anemic participants. Fifteen non-anemic was obtained from the participants of the Lyceum of the Philippines University Cavite and further testing was done on their hematocrit level to confirm their condition. A consent letter was given to each of the participants to assure that all of the information acquired in this research was strictly confidential. Non-anemic population bloods were examined with an aid of stained blood smear to determine the efficacy of crude Bougainvillea extract in color intensity and cellular morphology. The non-anemic population blood was examined with a stained blood test to determine the efficacy of the crude Bougainvillea extract in color intensity and cellular morphology.

2.3 Instrumentation

The authors used the form of observation. The observation form is filled out by two internal and two external microscopists in order to evaluate the trial and error and to avoid bias in the results. As a result, the color uptake of the blood test from the Bougainvillea crude extract compared to the Wright stain was examined. The main tool of the authors is the results of the comparison of the Bougainvillea crude extract, the Bougainvillea crude extract

with the methylene blue extract and the Wright stain through its staining properties by the differentiation of the nucleus and the color of the cytoplasm of the different blood cells. This was obtained by proper blood sample collection from non-anemic population and extraction of the Bougainvillea crude extract. In addition, the authors manipulated the light microscope for the observation of the staining properties by noting the nucleus and cytoplasm of different blood cells that served as criteria in distinction of extract against the Wright stain, hence the experiment acquired a good interpretation and analysis of the study. Furthermore, the evaluation of the data generated in the experiment was recorded in the observation form to show the differences of blood cell morphology to facilitate the interpretation of the data.

2.4 Extraction of Bougainvillea Bracts

The Bougainvillea spectabilis bracts were obtained from Pinagtipunan, General Trias, Cavite and was authenticated by Bureau of Plants and Industry for the validation of species. Bougainvillea spectabilis bract was washed with distilled water prior to extraction. Using analytical balance, Bougainvillea was weighed and crushed using mortar and pestle. Forty grams of Bougainvillea was macerated with 80 mL of 100% methanol which is then filtered and centrifuged. The extract obtained was stored in amber bottle [16]. The amber bottles prevented the crude extracts from possible chemical alterations by light and low temperature through degradation of anthocyanin. This was macerated for 72 hours upon addition of 100% methanol which is stored at room temperature.

2.5 Physical Properties Bougainvillea Bracts Crude Extract

The color of Bougainvillea bract crude extract was identified by looking down at the test tube against a white background under a good light source. Odor was also observed through cupping hands above the beaker and wafting of air towards the face. Determination of platelet was done through pH meter by soaking in the solution. Consistency of the crude Bougainvillea extract was determined by gentle aspiration into 5 mL pipette and allowing the extract to drop by gravity and the length of the thread formed was observed by the drops.

2.6 Blood Collection Procedure

Blood samples of the participants of the study were collected and no restriction to fasting was required among the subjects. The blood collection method used was the syringe method. As the vein was selected in the subjects arm, syringe was inserted and aspiration of blood was done by slowly pulling the plunger. The tourniquet was removed and put cotton and pressure to the site was applied. The needle was detached from the syringe and the blood was transferred to the tube by allowing it to flow on the side. Then, the tube was labeled

2.7 Hematocrit Determination

The blood from EDTA tube was collected with non-heparinized capillary tube and the bottom part of the capillary tube was sealed using clay. The capillary tube containing blood was centrifuged in micro hematocrit in 10,000 RPM for 5 minutes. Lastly, the value of hematocrit was determined through hematocrit reader by aligning the tube as bottom of plasma meets the line. The value of the hematocrit is read on the line with values where the RBC's meet the plasma by adjusting the ruler like assessor.

2.8 Blood Smear Procedure

The authors used the manual wedge method in blood smear preparation using a spreader slide and a film slide. A drop of blood was put on a clean glass slide at a point midway between the sides of the slide and a short distance from one end. Capillary tube was used to transfer a drop of blood from the EDTA tube to the slide. A drop of blood was about 2 mm in diameter which is approximately an inch away from the frosted area of the slide. The slide was placed on a flat surface, and in quick motion, the drop of blood was spread forming a

bullet shape occupying approximately 2/3 of the entire film slide. The smear was air dried and was labeled at the frosted edge with participant's number.

2.9 Staining Procedure

Each of the dried smears was stained by clipping the thick edge with a forceps by first fixing it in the 100 % methanol. For Wright stain procedure, the smear was dipped on the Wright stain for 5 to 10 seconds. The slide was then raised out of the coplin jar and the majority of the stain allowed to run off the slide. The slide was placed in jar containing deionized water to rinse off the excess stain. Excess fluid was wiped off from the back of the slide. The slide was placed upright on a paper towel with the feathered edge up and allowed to air dry.

For Bougainvillea staining procedure, it was fixed with methanol to preserve the blood smear. Then, it was dipped in Bougainvillea crude extract solution placed in the coplin jar for 10 minutes until the color has taken up by the smear and it was rinsed with water. On the other hand, for Bougainvillea crude extract with methylene blue staining procedure, it was fixed first with methanol followed by dipping it in the Bougainvillea crude extracted solution for 10 minutes, then rinsed with water and dip it with methylene blue for 1 minute. Next, excess stain was then washed off by tap water. When completely dried, the smear was examined under the microscope.

2.10 Microscopic Examination and Evaluation

The stained blood smear was placed under the light microscope; it was evaluated first in scanner, then in low power objective and high power objective. Cedar wood oil was dropped in blood smear for examination under oil immersion objective. Color uptake of Bougainvillea crude extract by red blood cells, white blood cells and platelet were observed. The cellular morphology and color intensity was graded through observation form. The color intensity was graded by noting whether it is acidic or basic. The cellular morphology was also graded by a rating of 0 to 5. The parameters for the determination of the cellular morphology of WBC, RBC and platelets are based on Table 1.

Table 1. Rating in Determination of the Cellular Morphology

Equivalent Rate	Observation
5	Very good- visibly stained RBC, platelets, and WBC with no morphological alterations
4	Good- visibly stained RBC, platelets, and WBC with no morphological alterations
3	Satisfactory/average- visible stained RBC, platelets and WBC with some morphological alterations
2	Poor- stained RBC, platelet and WBC only with morphological alterations present
1	Very poor- no observable morphology of cells
0	Non- staining

3. RESULT AND DISCUSSION

3.1 Physical Properties of Crude Bougainvillea Extract

Fresh Bougainvillea bract was macerated with 100% methanol for 72 hours to obtain its crude extract as shown in Figure 1. Right after the extraction process, physical properties were noted. Table 2 presents the physical properties of the crude extract as to the color, odor, pH and consistency. Extract was placed in a test tube against the white background for the observation of color. By looking down at the test tube, the extract appeared to be dark pink. The color was slightly lighter compared to its original bract prior to extraction process as this has undergone solvent dilution effect in which the solute concentration was reduced. On the other hand, the odor was noted as sweet honeysuckle-like. This was due to the bioactive substances of the Bougainvillea crude extract such as glycosides and reducing sugar. Measurement of the pH was checked using pH meter. The pH was slightly acidic (5.68) as Sabarudin et al. [17] stated that dyes produced high concentration of betacyanin at acidic pH (3 to 7) which wis responsible for the dark pink color as comparable to the Wright stain pH (6.4-6.8). According to Sabarudin et al. [17], Bougainvillea crude extract contains active substances like betacyanin which is responsible for its acidity. Furthermore, by aspirating the crude extract through a serological pipette and allowing it to flow through toward the tip of the pipette, it revealed a watery-like consistency. This was observed as fluidity of the extract as it was transferred directly to the receiving vessel without even forming a droplet.

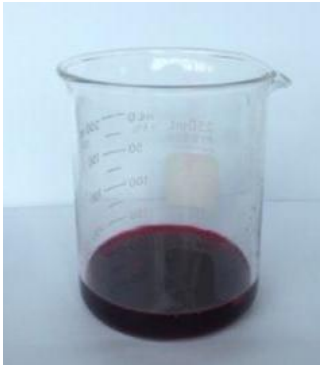


Figure 1. Crude Bougainvillea Extract

Table 2. Physical Properties of Crude Bougainvillea Extract

Physical properties	Observation
Color	Dark pink
Odor	Sweet honeysuckle-like odor
pH	5.68
Consistency	Watery-like consistency

The hematocrit values of the participants of the study were determined by microhematocrit manual methods which were taken as percentage of the whole blood total volume. As presented in Table 3, the hematocrit values are within the range of 37-47% which is within the normal reference range (35-54%) according to Bain [17]. This signifies that the population groups have a normal RBC volume.

Table 3. Hematocrit Level of the Population Group

Participants	Percent	Hematocrit

	Level (%)
1	46
2	45
3	46
4	45
5	45
6	47
7	45
8	47
9	40
10	37
11	44
12	37
13	45
14	37
15	47

3.2 Staining Efficacy of Bougainvillea Crude Extract and Bougainvillea Crude Extract with Methylene Blue

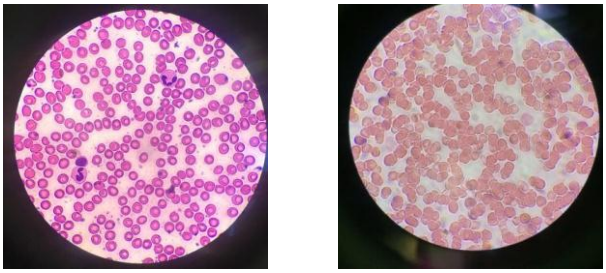
The staining ability of the Bougainvillea crude extract as potential alternative to Wright stain was blood smear preparation was depicted through Table 4 and 5, and Figure 2 and Figure 3. Comparison was made through differential uptake of the RBC, WBC and platelets between the crude extract alone, crude extract plus methylene blue against the standard staining using the Wright stain. The staining efficacy of the extract was rated based on the cellular morphology of the RBC, WBC and platelet and color intensity of the blood smear. Among the three blood cells, RBC cellular structure was satisfactory stained (average of 2.93) as halfway to the expected stained reaction of the Wright stain. RBC stained pinkish, less intense compared to the control as seen in Table 4. This however, still allowed the microscopic identification that those are red blood cells based on the size, shape and color.

White blood cells, on the other hand, as seen in the microscopic field (1000x), the morphologic identification and clarification of the cells was not applied because it stained very poorly (rating 1.40). Majority of the smears examined, appeared to be having the absence of the WBC as it did not taken up the stain color. Some smears where WBC was poorly stained, cytoplasm stained lightly pink and nuclear details were not clearly visualized. The cells could not also differentiate as to what type of WBC it was because of the absence of nuclear segmentation and granular appearance details.Lastly, thrombocyte or platelets among the smears obtained 0.87 rating. In the control slide, it appeared to be small, irregular, granular and retractile when the fine adjustment knob of the microscope was manipulated. In the smear stained with Bougainvillea crude extract, platelets have the poorest uptake of the stain. Even if the adjustment knob were manipulated, the characteristic refractive property of platelets was not manifested as well.

Table 4. Average Rating of Staining Efficacy of Bougainvillea Extract against the Control in terms of Cellular Morphology

Blood Cells	Average	Observation
Red blood cells	2.93	Satisfactory/ average-visible stained RBC with morphological alteration.
White blood	1.40	Very poor- no

cells		observable morphology of WBC
Platelet	0.87	Very poor- no observable morphology of platelet
N=15		



(a) (b)

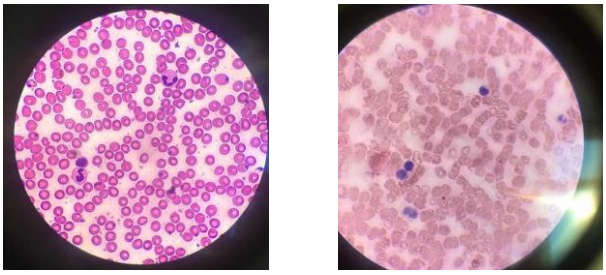
Figure 2. (a) Picture of blood smear stained with Wright stain (OIO 1000x) and (b) Bougainvillea extract (OIO 1000x).

Cellular morphology staining evaluations of different blood cells were also tested using crude Bougainvillea crude extract as primary stain and subsequently counterstained with methylene blue. Staining efficacy as compared to the Wright stain as control is presented in Table 5 and Figure 3. The combined Bougainvillea crude extract and methylene blue provided a satisfactory to good staining rating (3.20) with red blood cells. The counterstaining reaction showed an enhanced but slightly inferior intensity color of pink compared with the control stain however. The counterstaining of Bougainvillea crude extract and methylene blue provided a contrasting nuclear and cytoplasmic color among WBC. On some smears, WBC was clearly differentiated as whether mononuclear or polymorphonuclear because of differences in the color of the cytoplasm and nucleus. The staining uptake of different WBC with a rating of 2.68 (satisfactory), however, showed insufficient picture to identify the color of the granules of polymorphonuclear, which was critical for differentiation as whether they were neutrophil or eosinophil. For lymphocytes on the other hand, a very dark blue nuclear uptake was seen on some smears providing a better picture of the cell for its identification. Among platelets, a very light pink to colorless staining reaction characterized their staining uptakes. A very poor to poor rating (1.95) suggested that morphologic identification of the cell made by the microscopists unable to clearly identify the cell, which on some smears maybe mistaken already as just an artifacts.

Table 5. Average Rating of Staining Efficacy of Bougainvillea Crude Extract plus Methylene Blue against the Control in terms of Cellular Morphology

Blood Cells	Average	Observation
Red blood cells	3.20	Satisfactory/ average- visible stained RBC with morphological alteration.
White blood cells	2.68	Satisfactory/ average- visible stained WBC with morphological alteration.
Platelet	1.95	Poor- stained platelet with morphological

		alteration presents
N=15		



(a) (b)

Figure 3. (a) Picture of blood smear stained with Wright stain (OIO, 1000x) and (b) Bougainvillea crude extract plus methylene blue (OIO, 1000x).

3.3 Staining Efficacy of Bougainvillea Crude Extract and of Bougainvillea Crude Extract with Methylene Blue as to Color Intensity

Color intensity of the blood smear stained with Bougainvillea crude extract alone was rated and taken as percentage as to whether acidic or basic based on the color uptake of the entire smears. Table 6 shows the average percentage of blood smear from 15 non-anemic partcipants taken as triplicate smears on each subjects. Results clearly showed that roughly all the smears (99%) were acidic. Microscopic fields have the blood picture of pinkish to red color giving a monochromatic appearance among the cells identified as red blood cells. White blood cells were not recognized in the smears due to the predominant pink color. Maybe some of the cells have been mistaken as RBC already because normally, WBC stains with contrasting color pink cytoplasm and purple to blue nucleus. Platelets on the other hand were insufficiently graded as to their color intensity as platelets are absolutely non-recognizable among smears.

Table 6. Average Rating of Staining Efficacy of Bougainvillea Crude Extract against the Control in terms of Color Intensity

pH	Average (%)
Acidic	99
Basic	1
N=15	

As seen in Table 7, smears stained with Bougainvillea crude extract and subsequently with methylene blue suggest a percentage midway between acidity (61.5%) and alkalinity (38.5% staining result). The combined staining process provided a polychromatophilic color intensity that helped differentiate blood cells as RBC, WBC and platelets. This is in conformity with the Ehrlich's staining principle that acidic components of the cells takes up basic dyes and the basic cellular structure is stained with the acidic dye [19].

Table 7. Average Rating of Staining Efficacy of Bougainvillea Crude Extract plus Methylene Blue against the Control in terms of Color Intensity

pH	Average (%)
Acidic	61.5
Basic	38.5

N=15	

3.4 Significant Difference between the Staining Ratings of Bougainvillea Crude Extract and the Wright Stain as to Cellular Morphology

Determining the significant difference between the staining efficacy of Bougainvillea crude extract and the Wright stain is presented on Table 8 as to the cellular morphology of RBC, WBC and platelets in the stained smear. As manifested in Table 8, none of the stained blood cells produced comparable results with Wright stained smears. Since, statistically all the generated p- values were <0.05. This signifies that cellular uptakes of RBC, WBC and platelets showed lesser effectivity in allowing the cells to stained correctly as compared with the Wright stain.

Table 8. Wilcoxon W and Mann-Whitney U Test Result of Significant Difference between the Staining Ratings of Bougainvillea Crude Extract and the Wright Stain as to Cellular Morphology

Cellular Structure	Mean	p-value	Interpretation
RBC	2.78	0.014**	With Significant Difference
WBC	1.17	0.014**	With Significant Difference
Platelet	0.70	0.013**	With Significant Difference

Note:*-With no significant difference at >0.05 level **-With significant difference at <0.05 level

Establishing the staining capacity of Bougainvillea crude extract with methylene blue counterstain as comparable to Wright stain is obtained through the t-test. Based on Table 9, RBC got the closest mean rating to the standard stain. However, this is not enough to produce a blood smear stain based on the cellular morphology of the cells, with a statistically similar to the Wright staining reaction result. Despite that the RBCs both in the control smear and experimental smear showed a relative pink color with round to oval shape. For platelets and WBCs, smears stained with Bougainvillea crude extract with methylene blue showed a higher rating compared to the smears stained only with Bougainvillea crude extract alone. However, none of these cells even produced statistically non- significant results. This suggested that based on the evaluation rating given by four raters, Bougainvillea crude extract with methylene blue as a stain did not produced similar staining reactions with Wright stain for identification and classification of WBC, platelets and RBC based on their cellular morphology.

Table 9. Wilcoxon W and Mann-Whitney U Test Result of Significant Difference between the Staining Ratings of Bougainvillea Extract plus Methylene Blue and the Wright Stain as to Cellular Morphology

Cellular Structure	Mean	p-value	Interpretation
RBC	3.33	0.014**	With Significant Difference
WBC	2.68	0.014**	With Significant Difference
Platelet	1.92	0.014**	With Significant

			Difference

Note:*-With no significant difference at >0.05 level **-With significant difference at <0.05 level

4. CONCLUSION

Based on the results generated by evaluating the staining efficacy of Bougainvillea crude extract as potential alternative stain for Wright stain in blood smear preparation, the authors draw the following conclusions. The staining capacity of Bougainvillea in blood smear preparation is due to its bioactive substances, particularly, the betacyanin which was responsible for its acidity. It produced a pH of 5.6, which was near to the pH of the Wright stain. The ability of the extract to allow color uptake of the blood cells in blood smear preparation was made through maceration process using 100% methanol. This solvent was soluble with plant bioactive substance enhancing its effect to allow staining of blood cells. In addition, results generated through visual observation of the quality of the blood smears suggested that staining the smears with Bougainvillea crude extract alone have a poorest staining uptake with the platelets followed by the WBC. This extract preparation readily stains RBC but not as significantly the same as with the Wright stain. Likewise, staining results of the blood smears using Bougainvillea with Methylene Blue as a counterstain allows better morphological identification of WBCs because of the alternating pink-red and blue reaction which is ideally applied for WBC staining. The combined stains discriminate better the nucleus and cytoplasm of WBC more compared if only a single stain (crude extract alone) is used. This staining process is more effective in enhancing other cells as well, like the RBC and platelets compared to staining only with Bougainvillea crude extract. Statistically, the staining capacity of Bougainvillea as alternative to Wright stain showed a significant difference implying that this is not effective in staining the blood smear for morphology evaluation of the blood cells in manual CBC. However, the methods of evaluation done by the raters are subjective to their own perception and may vary among microscopists. The authors recommend further studies should be conducted using other methods of extraction of Bougainvillea pigments.

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