Anticoagulants are added to blood to stop the coagulation process both in vitro and in vivo. In the specific field of in vitro diagnostics, anticoagulants in hematology testing are commonly added to collection tubes to preserve blood samples in a state similar to their state inside the body (Banfi, Salvagno, & Lippi, 2007). Different types of anticoagulants are used depending on what test is being carried out. With the current trend of maximizing natural resources to minimize the waste products that could potentially harm the environment, studies are being done to determine natural sources of anticoagulants. One such possible source is Averrhoa bilimbi, locally known in the Philippines as kamias.

Averrhoa bilimbi, or its local name kamias, is found in the forests of Philippines and Southeast Asia. The fruit of the plant is commonly used as an ingredient for cooking but can also be used in treating wounds, rheumatism, venereal diseases, poisonous bites and beri-beri. The leaves can be used for cough and relief of rectal inflammation, while the flowers can be useful for coughs and thrush (Anitha, Geetha, & Lakshmi 2011). Kamias has a property to chelate metal cations. Based on a study by Daud, Hashim, and Samsulrizal (2013), the extract was effective in reducing the thrombophilic condition in rats. They extracted the fruit using ethanol and concentrated it using a rotary evaporator. The rats were treated with the fruit extract. A decrease in clot formation was observed.

Kamias’ ability to chelate metal cations is due to the presence of oxalic acid. Oxalic acid is an odorless, colorless, and strong organic acid that is naturally present in plants and vegetables. It is relatively stable below 189.5°C but it is affected by excess heat and highly reactive to oxidizing agents, metals and alkaloids, and forms as an oxalate when combined with soluble salts (Al-Wahsh, Wu, & Leibman, 2012). Oxalate, the conjugate base of oxalic acid, can chelate metal cations like Ca2+ and Mg2+ (McPherson & Pincus, 2017). Calcium is essential in the formation of a fibrin clot. Removing or inhibiting calcium would also inhibit fibrin clot formation (Turgeon, 2016).

There are currently multiple ways of extracting oxalate. Chromatography, rotary evaporation and manual extraction can be done to obtain oxalic acid. According to Al-Wahsh et al., (2012), extraction of oxalic acid can be done using hot or cold extraction using 2 N HCl and deionized water for both methods. In the material safety data sheet for oxalic acid found in the Science Lab website, it was mentioned that hot extraction from herbal plants yielded much more oxalate than cold extraction.

With these data in mind, kamias shows potential for serving as an anticoagulant for manual complete blood counts. Complete blood count or CBC is a routine laboratory test under the hematology division. A CBC includes red blood cell (RBC) and white blood cell (WBC) count, hematocrit, hemoglobin, WBC differential and RBC indices. RCC indices are calculated based on RBC count, hematocrit and hemoglobin. RBC indices include mean corpuscular volume (MCV), mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration (MCHC) (Rodak, Fristma, & Keohane, 2016).

CBC can be done manually or using automated machines. Manual counting of CBC requires the use of a hemocytometer, capillary tubes, microscope, dilution fluids, and pipette. According to Rodak et al. (2016), an automated method provides more accuracy and precision than manual methods and provides an early diagnosis and treatment of disease. However, the manual technique is more cost-effective and provides visualization of individual cell morphology.

EDTA (ethylenediaminetetraacetic acid) is the most commonly used anticoagulant in the hematology laboratory, and is the anticoagulant of choice for CBC because it preserves cell morphology and cell count (Eldin & Eldin, 2015). It chelates calcium and forms a complex that inhibits its action needed for coagulation (Kumar, Gousia, Anupama, & Latha, 2013).

Although EDTA can be used as the recommended anticoagulant for CBC, it can lead to erroneous results when blood is not immediately analyzed. This is based on studies done by Baffour, Quao, Kyeremeh, and Mahmood (2014) and Shrestha and Karki (2014). Artifactual changes in a blood smear like WBC vacuolation and platelet satellitism may occur if blood is stored in EDTA for more than two hours. Prolonged storage in EDTA for more than five hours can lead to changes in erythro-
cyte morphology. In addition, platelet clumping occurs when blood is stored in EDTA for four hours, giving a falsely low platelet count. Whenever these artifactual changes happen, it is necessary to recollect the sample and prepare the blood smear immediately.

**Objectives**

The objective of this study was to determine the use of *Averrhoa bilimbi* as an alternative anticoagulant for EDTA in manual complete blood counts.

**METHODOLOGY**

**Extract preparation**

Ripe fruits of *Averrhoa bilimbi* were harvested from a garden in Baranggay Malitlit, Santa Rosa, Laguna. Stalks and leaves were removed. The fresh fruits were washed with distilled water and dried; bruised or over ripe parts were removed. Juice collected after the fruits were processed in a blender and pressed was filtered using gauze to remove solid particles and was transferred into test tubes. The tubes with the extract were incubated in a water bath at 80°C for 30 minutes. The extract was then centrifuged at 4200 rpm for 10 minutes and the supernatant filtered using a Whatmann filter paper. The resulting extract was frozen until CBC could be performed.

**Sampling**

Six milliliters of whole blood were collected from 15 volunteers with no history of hematologic condition or current infection. The samples were divided into two aliquots with 3 mL whole blood per tube, where one tube contained a standard volume of EDTA as anticoagulant (lavender top) and the other 80 microliters of *A. bilimbi* extract as anticoagulant. The volume of extract added was based on a pilot test where different volumes of the extract were added to blood, and clotting time was determined. Tubes containing blood and extract that remained fluid were centrifuged to check for hemolysis. The lowest volume where no clotting occurred was chosen as the final volume used throughout the test. Manual CBC containing the following parameters was performed for both sets of tubes: RBC count, WBC count, hematocrit, hemoglobin level, neutrophil count, lymphocyte count, and mixed count.

**Complete Blood Count**

Red blood cell and white blood cell counts were obtained by diluting the blood using Thoma pipette and counting under the microscope using the Improved Neubauer hemocytometer. The Adam’s microhematocrit method was used for hematocrit and the WBC differential was based on the prepared peripheral blood smears stained with Hema-Quick stain and read at 1000x.

**RESULTS AND DISCUSSION**

The study demonstrated that crude extract from ripe *Averrhoa bilimbi* fruit has anticoagulant properties. Manual complete blood count with peripheral blood smear or film preparation was performed on blood with *A. bilimbi* extract, with EDTA-anticoagulated blood serving as control. Results of the RBC count, WBC count, hematocrit, hemoglobin, and the three-part differential were compared using the independent t-test to determine whether there is significant difference between the parameters tested.

**Effect of Averrhoa bilimbi Extract on Blood Cell Counts**

Based on the p value of the compared parameters of the independent t-test shown in Table 1, there was no significant difference in the results of the EDTA-anticoagulated blood, considered as the recommended anticoagulant for CBC, and whole blood using *A. bilimbi* extract as anticoagulant. The p values of RBC count, WBC count, hematocrit, hemoglobin, neutrophil count, lymphocyte count, and mixed count were all more than 0.05. This indicates that cell counts of blood with *A. bilimbi* extract is comparable to EDTA-anticoagulated blood.

<table>
<thead>
<tr>
<th>CBC Parameter</th>
<th>t</th>
<th>df</th>
<th>Sig</th>
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</thead>
<tbody>
<tr>
<td>RBC Count</td>
<td>0.196</td>
<td>28</td>
<td>0.846</td>
</tr>
<tr>
<td>WBC Count</td>
<td>1.461</td>
<td>28</td>
<td>0.155</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>-1.426</td>
<td>28</td>
<td>0.165</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-1.424</td>
<td>28</td>
<td>0.166</td>
</tr>
<tr>
<td>Neutrophil Count</td>
<td>0.579</td>
<td>28</td>
<td>0.604</td>
</tr>
<tr>
<td>Lymphocyte Count</td>
<td>0.979</td>
<td>28</td>
<td>0.869</td>
</tr>
<tr>
<td>Mixed Count</td>
<td>-0.749</td>
<td>28</td>
<td>0.631</td>
</tr>
</tbody>
</table>

p-value of <0.05 = significant
Effect of Averrhoa bilimbi Extract on Blood Cell Morphology

Blood smear evaluation was also performed to determine the effects of *A. bilimbi* extract on the morphology and distribution of red blood cells, white blood cells, and platelets right after the addition of the extract to the blood. In EDTA-anticoagulated blood, discernible changes in the blood cell morphology may be observed at approximately two hours from the time of collection. By 12 to 18 hours, the change in cell morphology becomes more apparent. Morphologic changes observed might include crenation or sphering in red blood cells; and vacuolation and nuclear budding in white blood cells. In a freshly prepared blood film from a patient with no underlying condition, all cells should be evenly distributed in the critical area, showing no clumps or aggregation. (Dacie & Lewis, 2015) In the current study, two slides were made and examined from all samples regardless of the additive used.

In evaluating the morphology of RBCs in blood with *A. bilimbi* extract, no rouleaux formation and agglutination was observed in the smear. However, two out of the 15 blood smears observed showed echinocytes (Figure 1) or crenation. Echinocytes, also known as burr cells, are red blood cells with spicules or small projections on the surface. Although it is a common artifact in peripheral blood films, echinocytes are frequently caused by use of hypertonic or alkaline solution and prolonged storage (Walker, Hall & Hurst, 1990). Comparison of the pictures of EDTA-anticoagulated blood film (Figure 2A) and *A. bilimbi* extract-anticoagulated blood film (Figure 2B) shows no echinocytes present in the blood film made from EDTA-anticoagulated blood. From the observations made, since only two out of the 15 samples exhibited echinocytes, this may be attributed to slight variations in the smearing or staining technique.

![Figure 1](image1.png)

*Figure 1. Photo showing echinocytes (arrow) present in peripheral blood smears of two samples with *A. bilimbi* extract.*

![Figure 2](image2.png)

*Figure 2. Comparison of pictures taken from peripheral blood films prepared from EDTA-anticoagulated blood (A) and blood with *Averrhoa bilimbi* extract (B).*

Upon evaluation of the WBC morphology, 10 out of 15 blood smears in *A. bilimbi* extract were observed to show more distortion of the cytoplasm and the appearance of vacuoles. Granulocytes exhibited more morphologic distortion compared to monocytes and lymphocytes. Cytoplasmic vacuolation (arrow) are present in the granulocytes shown in Figure 3 but lymphocytes in the right field appear morphologically normal. When comparing the slides prepared from EDTA tube and *A. bilimbi* tube, granulocytes from the EDTA tube exhibited almost no change in morphology (Figure 4). Cytoplasmic vacuolation, although one morphologic change observed in slides of patients with infection, may also be artefactual. Storage of more than two hours in EDTA may lead to autophagocytosis and is evidenced by the presence of small, distributed vacuoles in the cytoplasm. The appearance of vacuoles in the nucleus and cytoplasm especially in monocytes and neutrophils may be associated with loss of granules and nuclear swelling. (Rodak et al., 2016; McPherson & Pincus, 2017).
Figure 3. Pictures of peripheral blood smears showing morphologic changes in granulocytes of samples mixed with A. bilimbi extract.

Figure 4. Comparison of pictures of granulocytes in blood with EDTA (A) and blood with A. bilimbi extract (B) where granulocytes in the latter show cytoplasmic and nuclear vacuolization.

Platelets in peripheral blood smears prepared from fresh, whole blood should be evenly distributed in the critical area. Platelet activation during specimen collection, preparation or testing results in the formation of platelet clumps. (Rodak et al., 2016) Out of the 15 blood films prepared from samples with A. bilimbi extract, 11 showed platelet clumping (Figure 5B) that was not found in the control slide (Figure 5A).

Figure 5. Comparison of the platelet distribution of blood with EDTA (A) and blood with A. bilimbi (B) where blood with A. bilimbi shows platelet clumping.

CONCLUSION AND RECOMMENDATION

Oxalate anticoagulant has been reported to distort blood cell morphology faster than EDTA during storage. Degenerative changes associated with the use of oxalate in CBC includes shrinkage of red blood cells leading to lower hematocrit, nuclear swelling in white blood cells, and more rapid appearance of cytoplasmic vacuoles, abnormal segmentation of granulocytes, and loss of cell cytoplasm (McPherson & Pincus, 2017). However, in the current study, cell counts of blood with A. bilimbi extract and EDTA showed no significant difference. Morphologic changes in cells observed in the peripheral blood films prepared from blood with the extract may limit the use of the extract in morphologic evaluation of blood cells. Further study on the effect of other bioactive compounds present in A. bilimbi extract on blood cells is recommended.
REFERENCES


