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ORIGINAL RESEARCH ARTICLE

Kamias (*Averrhoa bilimbi*) Ethanolic Fruit Extract as an Alternative Anticoagulant for Complete Blood Count Testing and Peripheral Blood Smear

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***Abstract*— Ethylenediaminetetraacetic Acid (EDTA) is the additive used in complete blood count (CBC). *Averrhoa bilimbi* (Kamias) contains oxalate which can be an alternative source of the anticoagulant. This is an experimental research that aimed to assess the efficacy of Kamias ethanolic fruit extracts (KEFE) as an alternative anticoagulant to be tested in various CBC parameters and microscopical analysis of cells. Fresh Kamias fruit ws dried, pulverized, soak in ethanol for 2 days, and rotary evaporated. Comparisons of different concentrations of KEFE-treated blood (20, 30, and 40 uL) were compared with the EDTA-treated blood. The blood samples were collected with three volunteered participants with normal physical examination from Sta. Maria, Isabela. The identity and information accumulated will be disclosed to anyone. Thus, standard precaution was strictly monitored before, during, and after the procedure which also included the proper sanitation and disposal of waste. The clotting time of the different concentrations and EDTA-treated specimens were observed for more than 24 hours which proved its acticoagulation activity. Complete blood count testing was done using a semi-automated machine. The test parameters such as RBC, WBC, platelet, Hgb, Hct, MCV, MCH, MCHC were within normal range and it was analyzed using One-way ANOVA. Thus, the result showed no significant difference in the different parameters of the CBC test except in the platelet count. Microscopic analysis of blood smears treated with KEFE was conducted under the expertise of a certified pathologist. The observed blood smears exhibited distinctive platelet aggregation and disintegration patterns. Due to this, the KEFE has a comparable anticoagulation activity towards EDTA. The Kamias, KEFE, is a feasible alternative anticoagulant for CBC testing.**

Keywords*— *Averrhoa bilimbi, Ethylenediaminetetraacetic Acid (EDTA), Complete Blood Count, platelet aggregation, alternative anticoagulant

I. NTRODUCTION

Patient diagnosis is made easier through tests in clinical laboratories. This test includes a Complete Blood Count (CBC) test. According to the National Library of Medicine, CBC testing is a common blood test for routine check-ups. It is considered the most often requested diagnostic test in clinical laboratories (Wood et al., 2013). It also aids in the detection of disorders and diseases including infection, anemia, etc.

Ethylenediaminetetraacetic acid (EDTA) needs unclotted whole blood. It is stated in the study of Daud (2016), EDTA is a well-known anticoagulant that is most widely used in laboratories, which comes as sprayed liquids or dry crystal coats on ready-to-go tubes for blood extraction. It has certain advantages over other anticoagulants, making it ideal to use in hematological tests because it does not distort blood cells (Shrestha et al., 2014). In addition, it chelates calcium for the blood to inhibit clotting. Considering how frequently EDTA tubes are used in diagnostics, they can be costly. One of the disadvantages of using EDTA is platelet satellitosis. It happens when platelets are attached to the neutrophil which leads to a falsely decreased result. The conformational change in platelet membrane and antibody binding activity (IgG, IgM, or IgA) is caused by the calcium chelation of the said anticoagulant. If platelet satellitosis is encountered, sodium citrate is used to retrieve an accurate result. Thus, the alternative anticoagulant can be compared if the certain concentration of EDTA that causes erroneous results would have the same effect if the alternative anticoagulant is also used with the same concentration as the EDTA. Due to this, an alternative anticoagulant is essential.

Averrhoa bilimbi has been commonly used in traditional medicine for cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough, and hypertension (Daud, 2016). *A. bilimbi* is medicinally utilized by people who have renal

impairment (Alhassan, & Ahmed, 2016; Sa et al., 2019).

A. bilimbi has antihyperglycemic and anticoagulant effects; in consequence, it decreases the probability of thrombus formation in blood vessels (Daud, 2016). Kamias is an ethanolic fruit that contains oxalic acid that makes the anticoagulant effect of it (Hitalia et al, 2021). The ethanolic extract of *A. bilimbi* leaves had anticoagulant activity that is characterized by its ability to prolong blood coagulation time in both the extrinsic (PT) and intrinsic (aPTT) pathways. In *A. bilimbi*, Linn's antithrombotic and antioxidant actions in both normal and diabetic rats, several methanol/water extracts' total phenolic content (TPC) and total antioxidant capacity (TAC) were compared (Sitiawani et al., 2022). *A. bilimbi* extract as a source of natural thrombolytic activity was assessed to determine the thrombolytic activity of the *A. bilimbi*. The result shows an increase of 100ul to the clot of the positive control with 30,000 IU. 92.81% lysis of the clot was shown after 90 minutes of subsequent incubation at 37 degrees (Shrestha et al., 2014).

Mature Averrhoa fruits contain a lot of fiber and are known for their acidity. They are low in fat and high in antioxidants, potassium, calcium, phosphorus, iron, and other minerals. They are also excellent sources of vitamin C. Averrhoa fruits are ideal for usage as green vegetables for human consumption because of their traits and size. Furthermore, star fruits have adverse effects on uremic patients due to their high oxalic acid content (Shrestha et al., 2014). The said components can be used in assessing the anticoagulant activity of *A. bilimbi.*

This study sought to determine the effect of *A. bilimbi* on the various parameters in CBC testing. K3 EDTA tube was used as the control basis or standard to identify the appropriate concentration of *A. bilimbi* (KEFE) needed for the testing, and treatment of 20, 30, and 40 uL. The automated result was used in the CBC testing and for platelet assessment; it was assessed manually through peripheral blood smear (PBS). One-way ANOVA was used to determine the different concentration treatments that had a measurable effect in *A. bilimbi* and the commercially available anticoagulant.

II. METHODS

The researchers utilized an experimental research design using *A. bilimbi* (Kamias) fruit extract in blood specimens for the evaluation of its hematologic effect in vitro with positive control.

A. Participants of the Study

Three (3) blood donors aged between 19-25 years old from the vicinity of Sta. Maria, Isabela were included to provide blood specimen for this study. To make sure that the participants were physically fit, physical examination assessed by a physician was required prior the main testing. Consent was sought under the supervision of a lawyer. Thus, the researchers ensured their safety and followed the guidelines under the ethical standards. In choosing the participants, the criteria included no current/ underlying illness and must be a healthy individual. The approval of their participation depended on preliminary testing (physical examination) conducted by a physician.

B. Plant Sample Collection and Authentication

Two and one half (2 ½) kilograms of *A. bilimbi* fruit was freshly picked and collected along the vicinity of Sta. Maria. Both the ripe and unripe *A. bilimbi* fruit were used in the study. The *A. bilimbi* fruit sample was authenticated by a botanist affiliated to the Department of Agriculture. The collected fruit (2 kgs) was sundried for 48 hours to remove unnecessary moisture from the plant. Pulverization of the samples was performed using a blender. Half a kilogram per run and a strainer were used to filter the pulverized fruit to make sure that the dried sample was well pulverized (Hitalia et al., 2021).

C. Method for Extraction of A. bilimbi and Preparation of Treatments

The pulverized powder was wrapped in cotton and soaked in 70% ethanol (2L) for 2 days. After 2 days of soaking, the solution was placed for rotary evaporation at 60 °C. The extract was stored at room temperature until it was used. After the extraction, varying concentrations (20, 30, and 40 uL) were prepared. The Kamias Ethanol Fruit Extract was placed in a test tube and made ready for the placement of human blood specimens (Hitalia et al., 2021).

D. Method of Blood Specimen Collection

The blood sample was collected by a registered medical technologist. Venous blood sampling through the syringe method was used to extract the specimen (Liu, 2022).

E. Method of Blood Specimen Testing

a) Complete Blood Count. Specimen collected for CBC testing were tested right after collection. 3mL of the specimen was mixed with all the treatments (KEFE and positive control). An automated Hematology Analyzer machine was used.

b) Testing for Coagulation. This method was performed at 37 ° C by dropping 20, 30, and 40 uL of Kamias Ethanolic Fruit Extract in the tube and 3 mL of blood in the same tube and mixing it by inverting the tube. The sample sat, tilted, and observed every 30 minutes. Blood turning from a liquid to a gel is an indication of clot formation. The time when there was the first appearance of the clot formation was noted.

c) Clotting Time. A separate set of blood samples were also prepared with the different treatments. These were labeled and placed in the test-tube racks under room-temperature and were separately timed to monitor their clotting time.

d) Peripheral Blood Smear and Morphological Evaluation. A small drop of blood was obtained from the specimen mixed with the different treatments and placed on unlabelled slides for single blinding during the morphological evaluation. After drying the slides, the pathologist microscopically evaluated the morphology of the platelets (Prasad et al, 2022).

F. Data Analysis

The results of the various CBC parameters and the mean of platelet count from various concentrations were treated using

one-way analysis of variance (ANOVA) to determine the significant difference between the treatments as to CBC testing and platelet aggregation. The result may possibly develop as a substitute for the commercially prepared anticoagulants in a laboratory. It was used in determining the difference between the commercially available anticoagulant in terms of CBC testing (Automated) and Peripheral blood smear (manual). The clot formation time and the observations from the microscopic evaluation was briefly described.

G. Ethical Considerations

The researchers sought clearance from the University of Saint Louis Research Ethics Board with reference number 030-20204-04 before the conduct of this study.

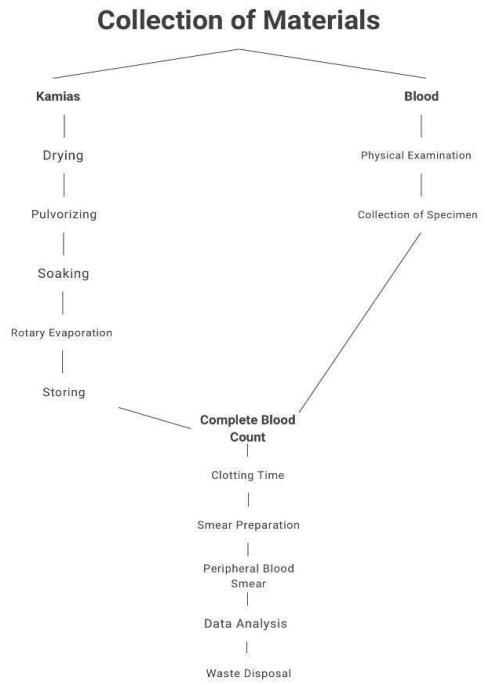

Fig. 1. Flow of procedures performed in the study

Procedure diagram served as the guide throughout the process of the experimental workflow.

III. RESULTS AND DISCUSSIONS

TABLE I. MEAN CLOTTING TIME OF BLOOD SPECIMENS EXPOSED TO DIFFERENT TREATMENTS

Treatments	Clotting Time	Qualitative Description
20µL KEFE	>24 hours	Above normal range
30µL KEFE	>24 hours	Above normal range
40µL KEFE	>24 hours	Above normal range
EDTA	>24 hours	Above normal range

The table shows that the clotting time of all blood specimens exposed to the different treatments is above the normal range of 8-15 minutes of sitting. This indicates that the different treatments show anticoagulation capacity.

TABLE II. COMPLETE BLOOD COUNT RESULT OF BLOOD SPECIMENS EXPOSED TO DIFFERENT TREATMENTS

CBC Parameter	Treatments			
	20µL KEFE	30µL KEFE	40µL KEFE	EDTA
WBC	10.56	10.48	10.50	8.95
Lymphocytes	3.66	4.01	3.74	2.24
MID	0.65	0.68	0.67	0.78
Granulocytes	6.26	5.78	6.26	4.92
Lymphocytes Percentage	35.20	38.97	36.07	37.03
MID Percentage	6.10	6.47	6.40	8.67
Granulocytes Percentage	58.70	54.57	57.53	54.30
Red Blood Cells	3.49*	4.54	6.02*	4.62
Hemoglobin	126.00	126.67	122.67	129.67
Hematocrit	36.90	37.57	23.92*	37.30
Mean Corpuscular Volume	82.23	82.87	82.90	80.90
Mean Cell Hemoglobin	28.07	27.90	28.40	28.00
Mean Cell Hemoglobin Concentration	341.33	336.33	340.33	346.33

**INDICATES ABNORMAL VALUES BASED ON REFERENCE VALUES*

The table above shows that majority CBC parameter values of the blood specimens exposed to the different treatments are within the normal range.

TABLE III. SIGNIFICANT DIFFERENCE IN THE CLOTTING TIME AND CBC PARAMETERS OF THE BLOOD SPECIMENS GROUPED ACCORDING TREATMENTS

Parameter	F-value	p-value	Decision
Clotting Time	0	0	Accept Ho
WBC	1.186	.375	Accept Ho
Lymphocytes	3.583	.066	Accept Ho
MID	.680	.589	Accept Ho
Granulocytes	.526	.677	Accept Ho
Lymphocytes Percentage	.098	.959	Accept Ho
MID Percentage	11.001	.003*	Reject Ho
Granulocytes Percentage	.197	.895	Accept Ho
Red Blood Cells	1.532	.280	Accept Ho
Hemoglobin	.076	.971	Accept Ho
Hematocrit	1.502	.286	Accept Ho
Mean Corpuscular Volume	1.428	.305	Accept Ho
Mean Cell Hemoglobin	.934	.468	Accept Ho
Mean Cell Hemoglobin Concentration	.630	.616	Accept Ho

**SIGNIFICANT AT 0.01 LEVEL*

The table above shows that the Clotting time and majority of the CBC parameters of the blood specimens treated with EDTA and the different concentrations of KEFE are significantly the same. This indicates that there are no significant deviations in these parameters. As such, the blood specimen treated with KEFE as anticoagulant yielded significantly similar results with those treated with EDTA.

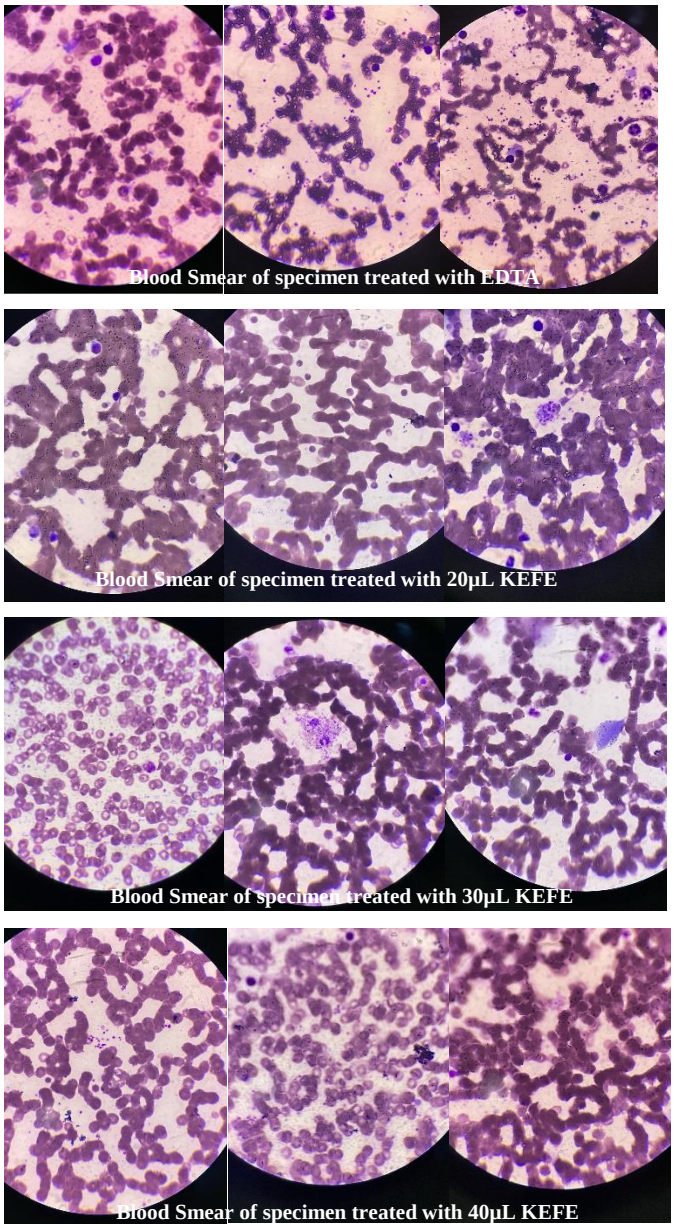

Fig. 2. Blood Smear of Specimen Exposed to Different Treatments

In can be seen in the figure above that the blood smear of treated with EDTA sample have red blood cells (RBCs) and discoid platelets, and few platelet aggregates. The blood smear of specimens treated with KEFE shows red cells rouleaux formation and clumps of platelet. Numerous stomatocytes and codocytes are also present. Few nucleated RBCs are also present.

This study shows the anticoagulation capacity of the Kamias Ethanolic fruit extract in blood samples. At 20 uL, 30 uL, and 40 uL Kamias Ethanolic Fruit Extract (KEFE) treated blood of three volunteers with no underlying diseases. Samples have remained unclotted even after 1440 minutes of observation and are comparable to EDTA.

These preliminary results indicate the potential anticoagulation activity of the Kamias. The Kamias contains high levels of oxalic acid (oxalate) with concentrations between 8.57 and 10.32 mg/g (Daud, 2016; Kausar et al., 2021). Oxalate is capable of chelating the calcium in the blood, thus, may inhibit the clotting capacity of the blood (Alhassan, & Ahmed, 2016; Keohane et al., 2019). Therefore, Kamias as a potential anticoagulant might be due to the presence of oxalic acid, the conjugate base is oxalate, from the fruit (Bhaskar & Shantarama, 2013; Daud, 2016).

Furthermore, the hematologic effects of the test groups were further evaluated using Complete Blood Count parameters that had been found that the majority of the parameters were comparable to EDTA-treated blood, with only platelet count showing a significant difference. Platelets were seen in KEFE-treated blood, but there was a significant difference compared to the EDTA-treated blood. Platelets in EDTA-treated blood were not aggregated while on the KEFE-treated blood from all of the concentrations, aggregations of platelets were seen.

Based on the results of the microscopic evaluation, the KEFE-treated bloods were comparable to the EDTA-treated blood. Red blood cells, white blood cells, and platelets were vividly seen. Although, there was a presence of RBC rouleaux, that was caused by the smearing technique of the medical technologist. It was also noted in the CBC results that there was sufficient amount of platelets in but it was too small to detect by the machine and can only be assessed through peripheral blood smear. And this was evident on the results of the microscopic evaluation. The difference was the means of the platelet count may be associated to its microscopic observation of the presence of aggregation. Oxalates as an in vitro anticoagulant may not inhibit platelet aggregation. With the undertaking that Kamias contains oxalates and the assumption that oxalate inhibits the chelation of calcium, oxalate might also have commenced the minor platelet aggregation, especially after exposure of the blood to the glassware (Bishop et al., 2022; Kausar et al., 2021).

Kamias contain high oxalic acid content. With this, it is contributory to highly acidic pH. The acidic pH of Kamias might have contributed to the distortion and vacuolation of cells. The pH of the Kamias extract was not determined and adjusted as part of the limitation of the study.

Kamias are easily grown and available locally. In this study, Kamias showed comparable hematologic effects with the commercially available anticoagulant. Much innovation can be generated from this natural resource by determining the optimum pH and adding a buffering agent. Possible optimization of the Kamias can also be further investigated by enhancing the concentration of extract against the whole blood. Other means of extraction may be utilized in order to extract

oxalic acid from the fruit to credit the anticoagulation property of the fruit to the oxalate component.

IV. Conclusion

The normal clot formation of blood in a tube without an additive can be carried out within 15 minutes. The blood specimen exposed to KEFE showed no coagulation even after 24 hours of standing. Results of the CBC of the blood specimens exposed to EDTA and KEFE showed ni significant deviation from the normal values. Moreover, the CBC results of the blood specimen treated with KEFE were found to be significantly the same with those treated with EDTA. However, in the evaluation through peripheral blood smear, it is found that the reason why the results of platelets in KEFE treated blood are decreased compare to the EDTA treated blood, is due to the disintegration, and it is noted that there was sufficient amount of platelets but it was too small to detect by the machine and can only be assessed through peripheral blood smear. It is concluded, therefore, that the ethanolic fruit extract of Kamias may be used as an alternative anticoagulant specifically for CBC testing. However, further study must be done in order to prevent the possible platelet aggregation.

V. Recommendations

The alternative anticoagulant proved its effectiveness in the different parameters under CBC testing but the presence of platelet aggregation and decreased platelet count were noted. Different concentration of KEFE must also be tested aside from the concentration 20, 30, and 40 uL. Different extraction method must be explored, as well as the smearing technique.

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References

Alhassan, A. M., & Ahmed, Q. U. (2016). Averrhoa bilimbi Linn.: A review of its ethnomedicinal uses, phytochemistry, and pharmacology. Journal of Pharmacy& Bio allied Sciences, 8(4), 265–271. https://doi.org/10.4103/0975-7406.199342

Bhaskar, B., & Shantaram, M. (2013). Morphological and biochemical characteristics of Averrhoa fruits. *International journal of pharmaceutical, chemical and biological sciences*, *3*(3), 924-928.

Bishop, R. C., McCoy, A. M., Kemper, A. M., Stewart, R. M., & Wilkins, P. A. (2022). Short-term administration of flunixin meglumine or firocoxib does not alter viscoelastic coagulation profiles in healthy horses. *Journal of the American Veterinary Medical Association*, *260*(15), 1963-1966. https://doi.org/10.2460/javma.22.08.0367

Daud, N. (2016). *The acute toxicity of Averrhoa bilimbi Linn leaves and fruits ethanolic extracts and their antidiabetic and anticoagulant activities* (Doctoral dissertation, Universiti Teknologi MARA). Retrieved from https://ir.uitm.edu.my/id/eprint/17895/2/TM_NURAFIFAH%20DAUD%20AS%2016_5.pdf

Hitalia, J. P., Bringuelo, Y. J., Jordan, J. H., Martinez, E., Leba Jr, R., Vadez, A. G., ... & Paculanan, A. (2021). Kamias (Averrhoa Bilimbi), starfruit (Averrhoa Carambola), and karamay (Phyllanthus Acidus) fruit extract as alternative stain remover. *ASEAN Journal of Science and Engineering*, *1*(1), 19-22. Retrieved from https://ejournal.kjpupi.id/index.php/ajse/article/view/269

Kausar, S., Fateen, T., & Chaudary, H. T. (2021). Frequency of Causes of Spurious Platelets Count on Routine Complete Blood Count by an Automated Hematology Cell Analyser. *Liaquat Medical Research Journal*, *3*(1). Retrieved from http://121.52.154.206/index.php/LMRJ/article/view/585

Keohane, E. M., Otto, C. N., & Walenga, J. M. (2019). *Rodak's Hematology-E-Book: Rodak's Hematology-E-Book*. Elsevier Health Sciences. Retrieved from https://books.google.com/books?hl=en&lr=&id=IoGJDwAAQBAJ&oi=fnd&pg=PP1&dq=Rodak+2012+clotting&ots=1RTzFzeu6-&sig=42cNnlIYE3ItOzKVCtsst58lc04

Liu, Y. T., Lee, M. H., Lin, Y. S., & Lai, W. L. (2022). The Inhibitory Activity of Citral against Malassezia furfur. *Processes*, 10(5), 802. https://doi.org/10.3390/pr10050802

Prasad, V., Sharma, D., & Verma, S. L. (2022). Peripheral Blood Smear Examination: An Overview. *Webology*, *19*(2). Retieved from https://www.webology.org/data-cms/articles/20220219114530amwebology%2019%20(2)%20-%20234%20pdf.pdf

Sá, R. D., Vasconcelos, A. L., Santos, A. V., Padilha, R. J. R., Alves, L. C., Soares, L. A. L., & Randau, K. P. (2019c). Anatomy, histochemistry and oxalic acid content of the leaflets of Averrhoa bilimbi and Averrhoa carambola. *Revista Brasileira de Farmacognosia*, 29(1), 11– 16. https://doi.org/10.1016/j.bjp.2018.09.005

Shrestha, A., & Karki, S. (2014). Evaluation of EDTA induced pseudothrombocytopenia and the effect of alternative anticoagulants. *Journal of Pathology of Nepal*, *4*(8), 626-629.

Sitiawani, M., Azizah, Z., Asra, R., & Chandra, B. (2022). Phytochemical of Some Plants with Anticoagulant. *Asian Journal of Pharmaceutical Research and Development*, *10*(4), 55-63. https://doi.org/10.22270/ajprd.v10i4.1157