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Effect of *Citrofortunella microcarpa* (Calamansi) Peelings on Whole Blood Coagulation Using Blood Samples from Albino Mice

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SJRR and LGT designed the study. Authors ICNR, SJRR, LGT, KAR and MAMMS wrote the protocol and first draft of the manuscript and performed the experimental procedures. Author ICNR managed the literature searches. Author LGT organized the results. Author SJRR performed the statistical analysis. Authors ICNR, SJRR and LGT wrote the discussion of the study. Authors ICNR and SJRR revised the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Citrofortunella microcarpa, locally known as calamansi in the Philippines, is an intergeneric hybrid between *Citrus reticulata* and *Fortunella japonica.* This fruit is widely cultivated in the Philippines for its fruit juice as an abundant source of vitamin C and as a condiment in many local foods in the country. Sadly, only the pulp is needed for squeezing while the peels are thrown after extracting the juice. Previous studies revealed that the peels of Citrus, as member of the Rutaceae family, can synthesize both coumarins and furanocoumarins wherein their derivates are used as oral anticoagulants which can inhibit vitamin K from functioning as a cofactor in the hepatic synthesis of the vitamin K-dependent coagulation factors II, VII, IX, and X. In this study, the extract of calamansi peelings were proven to have an anticoagulant property on blood samples from albino mice. This

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study will pave the way for scientists to allot time in studying calamansi peelings for it may be another source of medicine to help patients who are prone to have stroke, myocardial infarction, and other blood clotting diseases.

Keywords: Calamansi peelings; Citrofortunella microcarpa; citrus; rutaceae; anticoagulant; whole blood coagulation.

1. INTRODUCTION

Calamansi, scientifically known as *Citrofortunella microcarpa*, is a famous source of staple fruit juice in the Philippines [1,2]. This fruit is an intergeneric hybrid between *Citrus reticulata* or mandarin orange and *Fortunella japonica* or kumquat [3,4]. It is locally known as a good source of vitamin C which makes it very abundant. For this main reason, calamansi is widely cultivated in the Philippines for its fruit juice. Aside from that, this fairly sour fruit is also added as a condiment in many local foods in the country [1,2]. Sadly, only the pulp is needed for squeezing while the peels are thrown after extracting the juice. The medicinal use of the peel is still unknown to many Filipinos.

Citrus, as a member of the Rutaceae family, can synthesize both coumarins and furanocoumarins [3]. Previous studies revealed that citrus peel contains larger diversity and higher concentrations of coumarin or furanocoumarin than the pulp of the same fruits [5,6,7]. Coumarin derivatives are used as oral anticoagulants that inhibit vitamin K from functioning as a cofactor in the hepatic synthesis of the vitamin K-dependent coagulation factors II, VII, IX, and X [7,8].

Calamansi fruit is believed to have a compound coumarin which is now scientifically being studied as an anticoagulant. Coumarins comprise a very large class of compounds found throughout the plant kingdom [9]. They are found at high levels in some essential oils, such as cinnamon bark oil, cassia leaf oil, and lavender oil [10]. Aside from Citrus fruits, coumarin is also found in other fruits like bilberry and cloudberry, as well as in other foods and beverages such as chicory, green tea, and herbal tea [6,11]. Most coumarins are found in higher plants, with the richest sources being the Rutaceae. Although distributed throughout all parts of the plant, the coumarins are concentrated at the highest levels in the fruits, followed by the roots, stems, and leaves [9,12].

Family Rutaceae which includes genus *Citrus* have numerous species and a notable species studied for antimicrobial properties is *Citrus limon* L. Burm. f. which is commonly found in South Asia [7]. Its properties have been utilized for some specific venom as remedy because of the platelet inhibitory actions together with hypercholesterolemic effect [13,14]. The anticoagulant and thrombolytic actions of *Citrus limon* on blood parameters were not yet thoroughly researched, and studies involving in-vivo and in-vitro revealed to help investigate if there would be an impact on anticoagulation and coagulation factors. Some in-vitro tests showed significant increment in thrombin time by *Citrus limon,* although fibrinogen was diminished in comparison to control, prothrombin time was not influenced altogether [7].

An *in-vivo* study using *Citrus limon* was done in rabbits of three groups. There were notable changes seen in the blood parameters like erythrocytes, hemoglobin and mean corpuscular hemoglobin. Thrombin time and draining time were obtained and there was an increase in protein C and thrombin antithrombin complex. These results might be due to thrombin inactivation that would diminish fibrinogen concentration and would repress aggregation of platelets. *Citrus limon* has demonstrated maximal anticoagulant effect which would suggest that it has an anti-thrombin component that could help prevent thrombosis [7].

Coumarin and its derivatives are known to be components of principal oral anticoagulants. Its action is to impede multiple stages in the coagulation cascade. Fibrinolytic agents act to lyse thrombi that causes different pathology in the body. Coumarins are known to be competitive inhibitors of vitamin-K in prothrombin biosynthesis. Coagulation cascade would depend on the conversion of prothrombin to thrombin in a very crucial manner [9,15,16].

On the other hand, heparin has been used as an anticoagulant for a long time and is used as a treatment and prevention of deep vein thrombosis and arterial thromboembolism. It is also given in cases of heart attacks and unstable angina. It acts as an anticoagulant and blocks the clotting cascade [17,18].

Coagulation, also known as clotting, is the process by which blood changes from a liquid to a gel eventually leading to clot formation. It eventually results in hemostasis, which is the cessation of blood loss from a damaged vessel that is subsequently followed by repair. Mechanisms of coagulation would involve activation, adhesion and platelet aggregation together with deposition and fibrin maturation [19]. After bleeding has ceased and healing has started, the body will respond by breaking down and removing the formed blood clot. Excessive blood clot means that blood clotting formation is rapid or may not break down properly and subsequently has the tendency to travel through the body resulting to a limited or blocked blood flow. These clots can form in, or travel to, the arteries or veins in the brain, heart, kidneys, lungs, and limbs that can increase the likelihood of heart attack, stroke, damage to organs, or even mortality [15,19]. In this experiment, coagulation was tested with calamansi peelings and heparin.

In this regard, this study aimed to answer the following research questions: (1) do calamansi peelings have anticoagulant property on whole blood sample from albino mice; and (2) is there a difference in the clotting time between the 50% and 100% concentration of calamansi peelings?

2. MATERIALS AND METHODS

The aims of this experimental study are to determine the anticoagulant property of calamansi peelings on whole blood sample from albino mice and to differentiate the clotting time between half and pure concentration of calamansi peelings.

Fresh peelings of calamansi were chopped into pieces and were eventually subjected into boiling (infusion and decoction). The concentration of the calamansi peelings such as water were prepared as 50% and 100%. Blood samples drawn from the tails of 10 albino mice were collected and placed in their respective slides. The sample slides were prepared namely: slide A for blood only; slide B for blood with control (heparin 20 µL/mL); slide C for blood with calamansi extract 50%; and slide D for blood with calamansi extract 100%. Using lancet, blood samples were checked every 30 seconds to observe if there is fibrin formed. The blood samples were continued in testing using lancet. If there is fibrin formed, the time was recorded as the clotting time.

This experiment was done in the Pharmacology Laboratory of the School of Medicine of Centro Escolar University with the approval and supervision of the Faculty of the Department of Pharmacology.

3. RESULTS AND DISCUSSION

Based on the results, the mean of slide A (blood only) is 472.20 seconds or 7.87 minutes, the mean of slide C (blood with 50% extract) is 1,042.50 seconds or 17.38 minutes, and the mean of slide D (blood with 100% extract) is 1,399.20 seconds or 23.32 minutes. Therefore, the 50% extract of calamansi peelings has faster anticoagulant effect compared to the 100% extract of calamansi peelings. In relation, this finding also suggests that the 100% extract has slower anticoagulant effect than the 50% extract. Although heparin is still the best among the three anticoagulant treatments in this experiment, the findings suggest that both 50% extract and 100% extract of calamansi peelings proved to have an anticoagulant property since the blood coagulated much later than the slide A which has no treatment

Table 1. Results of the study

Albino mice	Slide A blood only (minutes)	Slide B blood with heparin (minutes)	Slide C blood with 50% extract (minutes)	Slide D blood with 100% extract (minutes)
1	8:00	-	18:00	27:00
2	7:33	-	12:27	15:08
3	5:28	-	17:00	20:00
4	6:20	-	18:02	24:00
5	9:24	-	20:01	25:03
6	8:05	-	16:57	26:05
7	6:58	-	15:08	23:08
8	7:05	-	13:33	19:25
9	9:28	-	22:12	26:28
10	10:19	-	23:24	27:08
Mean	7.87	-	17.38	23.32

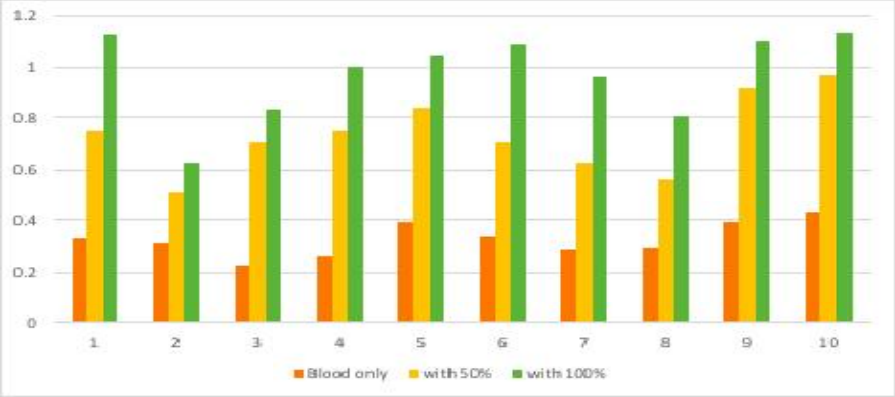

Fig. 1. Bar graph of the results of the study

	A	B	C	D
1	480	0	1080	1620
2	453	0	747	908
3	328	0	1020	1200
4	382	0	1082	1440
5	564	0	1081	1503
6	485	0	969	1565
7	418	0	908	1383
8	425	0	801	1165
9	568	0	1333	1588
10	619	0	1404	1620
n	10	10	10	10
$\bar{X}$	472.200	0.000	1042.500	1399.200
s	90.581	0.000	207.943	237.849
$\bar{X}_{ave}$	728.475			

source	df	SS	MS	F	P-value
treatments	3	11448364.275	3816121.425	141.3152	0.0011
error	36	972155.700	27004.325		
total	39	12420519.975			

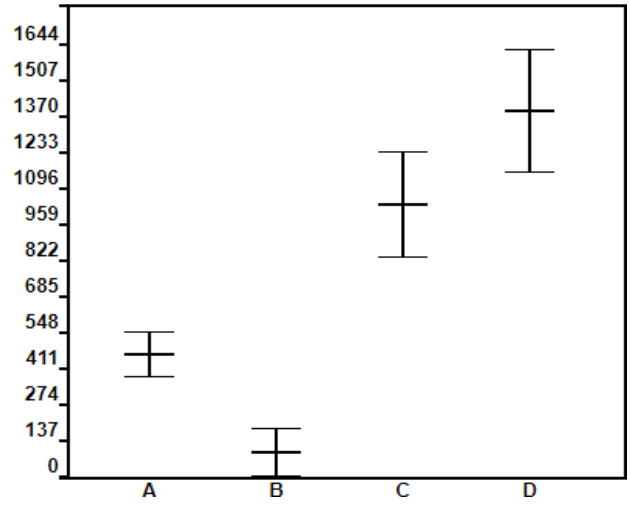

Fig. 2. Statistical analysis of the results of the study

Based on the statistical analysis, the null hypothesis (Ho) which states that calamansi peelings have no anticoagulant property on blood sample from albino mice is therefore rejected since the P-value is 0.0011 which is statistically interpreted as highly significant. In this regard, the calamansi peelings have anticoagulant property on blood sample from albino mice.

The results of this study confirmed the findings from the previous studies on Citrus family. As evidenced by the previous studies, fruits from the Citrus, especially the peels [6], demonstrated maximal anticoagulant effect, which recommend that they have anti-thrombin component and could counteract thrombosis assuming a cardio-protective job [5,7]. As a result, excessive blood clotting can be prevented, which thereby prevents clots to travel to the brain, heart, kidneys, lungs, and extremities, which can cause heart attack, stroke, damage to the organs, or death [7,19].

4. CONCLUSION

Calamansi is mainly used for its pulp and juice while the rest of the fruit such as covering of pulp segment, seeds, and peelings are considered waste. Since calamansi belongs to Citrus family, it can possibly be used as an anticoagulant. If its potential consumption as phytomedicine is explored and investigated, it can also help in reduction of environmental pollution since the peelings will no longer go into waste after extracting the juice and pulp.

This study, therefore, concludes that the peelings of calamansi, as a member of Citrus family, has coumarin derivatives that makes it effective anticoagulant. The result of this study may be useful to people who are prone to have stroke, myocardial infarction, and other blood clot formation diseases which is common nowadays.

The authors are hoping that this study will pave the way for experts in the field of biological and medical sciences, specifically in pharmacognosy, to allot time in experimenting calamansi peelings for it may be another source of medicine to help many people. Further studies and experiments on calamansi peelings are recommended.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This experiment was conducted in accordance to the ethical norms approved by the institution.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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