



## Hemostatic activities of methanol leaf extracts of *Croton megalocarpus* Hutch and *Lantana camara* Linn

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### ABSTRACT

The effects of *Croton megalocarpus* Hutch and *Lantana camara* Linn plants leave on blood coagulation has not been scientifically tested despite their common use in bleeding control. The current study investigates the effects of the plants' leaves extracts on mice plasma coagulation time. Leaves of *C. megalocarpus* (H.) and *L. camara* (L.) were harvested, washed, air dried, crushed, extracted in absolute methanol and concentrated to dry powders. Preliminary qualitative phytochemical screen of both extracts was done. A total of 55 mice plasma samples were mixed with solutions of leaves extracts of both plants, before prothrombin time (PT) and activated partial thromboplastin time (aPTT) were determined. Data was analyzed using one – way ANOVA. Phytochemical screen on separate extracts of *C. megalocarpus* (H.) and *L. camara* (L.) revealed the presence of tannins, phenols, cardiac glycosides, and terpenoids. Moreover, *C. megalocarpus* (H.) extract contained steroids and flavonoids while that of *L. camara* (L.) had saponins. Solutions of the various concentrations of methanol leave extracts of *C. megalocarpus* (H.), *L. camara* (L.) and leaves blend extracts from both plants reduced aPTT and PT significantly ( $p < 0.05$ ) when mixed with normal mice plasma. Significant reduction of aPTT and PT of normal treated plasma was associated with the activity of tannins, phenols, flavonoids and saponins detected in the plant extracts.

### INTRODUCTION

Vessels in the vascular network are lined with endothelium whose smooth surface resists the adhesion of blood cells on its surface. There exists an innate emergency system called hemostasis, which utilizes proteins and mucopolysaccharides to minimize blood loss when the integrity of blood vessels is breached due to injury or surgery [1]. Hemostasis involves an elaborate interaction between platelets and proteins leading to the formation of a fibrin clot, sealing the injured site [2]. The mechanism involves two distinct phases: the primary and secondary hemostasis which take place simultaneously [3, 4]. Primary hemostasis results in vasoconstriction at the injury site, arresting blood loss following vascular spasm [3]. Following the activity of Von Willebrand factor, platelets are activated and

attracted to the site of injury during primary hemostasis [5]. The activated platelets also bind to each other to form a temporary platelet plug over the bleeding site, occluding the injured surface [3]. Secondary hemostasis results in the production of fibrin fibers over the temporary platelet plug formed during primary hemostasis [4]. The secondary phase of hemostasis involves the coagulation cascade, which is made up of three distinct but interrelated branches; the intrinsic, extrinsic and the common pathways [1, 5].

Each pathway is made up of a group of inactive coagulation enzymes called zymogens [6]. In case of injury, the exposure of the endothelium leads to sequential activation of initial zymogens in the cascade into serine proteases [6]. The serine proteases formed act as catalysts for the activation of the next zymogen chain, converting it into a serine protein,

ultimately resulting in the conversion of fibrinogen into fibrin fibers [2]. Major injuries may overwhelm hemostasis leading to massive blood loss and death [3].

Blood loss control is an important factor in the survival following vascular injury [4]. Between 30 and 40% of trauma related deaths occurs within the first few hours after injury with about 10% of them being preventable [7]. In Kenya, road traffic accidents form 61.7% of the total disease burden [8]. This makes bleeding associated with traumatic injury one of the top ten leading causes of mortality in Kenya [9]. Hemostatic agents control bleeding by enhancing coagulation through the stimulation of fibrin formation or inhibition of fibrinolysis [10]. The ideal hemostatic agent needs to be inexpensive, easy to use, and capable of extended storage in a wide range of temperatures, ability to stop bleeding in 2 minutes and void of mixing or pre-application preparation [5, 11,13, 14]. Currently, no conventional hemostat is perfect [10]. Conventional hemostatic agents range from coagulation proteins to complex carbohydrates [11]. These include platelet sealants, oxidized cellulose, thrombin, fibrin sealants, microfibrillar collagen, and gelatin [11].

Conventional agents are associated with various adverse effects. Hemostats prepared from human sources which include thrombin and fibrin sealants carry risks of transmission of blood-borne viruses. [12, 15]. In addition, products derived from bovine sources are known to elicit immunologic reactions to users [11, 15]. Gelatin swells when applied to bleeding sites, making its use in body cavities to be undesirable [11]. Furthermore, oxidized cellulose was found to form fibrous scars after wound healing [11]. Besides unwanted effects, thrombin-based hemostats like fibrin sealants and microfibrillar collages are expensive to manufacture [11,12, 16]. Currently, the world is experiencing a surge in interest, acceptance and use of herbal remedies, in both developed and developing countries [17]. However, herbal remedies need to be evaluated for their effects to avoid relying on traditional beliefs and assumptions [18].

Traditionally, sap from fresh leaves of *Croton megalocarpus* (Hutch) is used topically to arrest bleeding and wound healing [19]. Paste from fresh leaves of *Lantana camara* (Linn) is applied topically to stop bleeding from cuts [20, 21, 22]. Therefore, this study was designed to evaluate the effects of methanol extracts of *C. megalocarpus* (MECM.) and *L. camara* (MELC) on plasma coagulation. MECM and MELC

extracts were both found to reduce coagulation time of mice plasma involving both intrinsic and extrinsic pathways *ex vivo*.

## MATERIALS AND METHODS

### Extract preparation

Guided by folk literature, fresh leaves of *C. megalocarpus* (H.) and *L. camara* (L.) were collected with help of local herbalists at Mbooni, Makueni County. The leaves were transported to the study site at Biochemistry laboratories, situated at Kenya Methodist University School of Medicine and Health Sciences, Meru campus. The samples were verified and authenticated by a paratonomist. Voucher specimens were deposited at the United States International University - Africa's School of Pharmacy and Health Sciences herbarium (*C. megalocarpus* (H.) MUINDI.H/014; *L. camara* (L.) MUINDI.H/013).

Leaves were washed, air dried under shade for 14 days and milled to fine powders using an electric mill (Christy and Norris Model 8). Separate powders of *C. megalocarpus* (H.) and *L. camara* (L.) leaves were weighed using an analytical balance (Citizen Scales, Edison, USA) and placed in separate beakers then mixed with absolute methanol (ratio of 150 grams of powder to 1.5 liters of methanol) and allowed to extract for 48 hours with agitation. Leaves from both plants were blended (75g of *C. megalocarpus* (H.) powder and an equal weight of *L. camara* (L.) powder) before extraction in 1.5 liters of absolute methanol. Following filtration with Whatman filter paper number 1, the extracts were then concentrated by use of a rotary evaporator (Stuart RE400, Stuart, Germany) at 45°C under pressure to dry powders.

### Phytochemical analysis

The qualitative phytochemical analysis of MECM and MELC extracts were carried out by the standard methodology for testing the alkaloid, phenols, tannins, saponins, anthraquinones, flavonoid, steroids, terpenoids, cardiac glycosides, carbohydrate and proteins [23].

### Extracts and control preparation

Powders of MECM and MELC extracts were weighed using an analytical balance (Citizen Scales, Edison, USA) and then dissolved in distilled water to make

concentrations of 0.25, 0.5 and 1 mg/ml. A volume of 0.1 ml of dimethyl sulfoxide (DMSO) was added to each extract solution to enhance solubility. An aqueous solution of the reference drug (*StopsBleeding*<sup>TM</sup>, CoAg Medical, Minnesota, USA) was prepared to a concentration of 0.5 mg/ml. Normal control was prepared by adding 0.1ml of DMSO to 4.90 ml of distilled water.

### Plasma sample collection and preparation

The approval for use of laboratory animals for the research was obtained from the National Commission for Science, Technology, and Innovation (Reference number NACOSTI/P/20/4729). Fifty-five adult (4-month-old), laboratory-bred Swiss white albino mice, weighing between 200 and 300 grams were obtained. They were allowed to acclimatize in the laboratory for 1 week with unlimited access to water and rodent pellets. Blood was collected from all mice by cardiac puncture into labelled plastic tubes containing sodium citrate anticoagulant (Vacutainer, BD), making a ratio of 9:1. Samples were immediately centrifuged for 5 minutes at 7000rpm using Eppendorf 5427R refrigerated centrifuge (Eppendorf AG, Germany). Plasma was separated into cryogenic plastic tubes and stored at -25°C awaiting evaluation.

### Experimental design

The study used a randomized controlled *ex vivo* experimental design where plasma of 55 mice were randomly placed into 11 treatment groups with 5 samples each. Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) tests were used to evaluate hemostatic effects of MECM, MELC and MECM - MELC blend leaves extracts and controls on treated mice plasma following standard method [24].

Group I (normal control) plasma samples were mixed with distilled water (vehicle) and dimethyl sulfoxide. Groups II (positive control) plasma samples were mixed with the reference hemostatic drug (*StopsBleeding*<sup>TM</sup>), at a concentration of 0.50 mg/ml. Groups III, IV and V samples were mixed with MECM extract solutions at concentrations of 0.25, 0.50 and 1 mg/ml respectively. Groups VI, VII and VIII samples were mixed with MELC extract solutions at concentrations of 0.25, 0.50 and 1 mg/ml respectively. Groups IX, X and XI samples were mixed with blend methanol leaves extracts of MECM-MELC extract at

concentrations of 0.25, 0.50 and 1 mg/ml respectively as shown in table 1.

**Table 1.** Experimental design for the determination of hemostatic effects.

Group	Treatment	Samples (n)
I (Normal control)	Distilled water + 1% DMSO	5
II (Positive control)	<i>StopsBleeding</i> <sup>TM</sup> * (0.50 mg/ml)	5
III	MECM (0.25mg/ml)	5
IV	MECM (0.50 mg/ml)	5
V	MECM (1 mg/ml)	5
VI	MELC (0.25mg/ml)	5
VII	MELC (0.50 mg/ml)	5
VIII	MELC (1 mg/ml)	5
IX	MECM-MELC (0.25mg/ml)	5
X	MECM-MELC (0.50 mg/ml)	5
XI	MECM-MELC (1mg/ml)	5

MECM (methanol leaves extract of *Croton megalocarpus*), MELC (methanol leaves extract of *Lantana camara*), MECM-MELC (methanol blended leaves extract of *Croton megalocarpus* and *Lantana camara*). \* The reference drug.

### Effects of extracts on prothrombin time

On the Start4 coagulometer user menu, PT test mode was selected, and the sample identification number entered. A set of three test cuvettes were obtained and analysis metal balls added. A volume of 50 µL of the respective extract/ control solution was added into three cuvettes containing analysis metal balls [24]. A similar volume of platelet poor plasma was then added and mixed with the extract/ control. The mixture was incubated for 5 minutes at 37°C in Start 4 (Diagnostica Stage, France) coagulometer incubation channels [24]. Pre-incubated Neoplastine CI Plus (100 µL) was added using the equipment timer-linked pipette then coagulation detected automatically, and results printed. The average from the three test runs for each sample was calculated and recorded [24].

### Effects on activated partial thromboplastin time

aPTT test mode was selected by navigating Start4 coagulometer user menu and sample identification number entered. Analysis metal balls dispensed into three test cuvettes. A solution of respective

sample/control solution (50  $\mu$ L) was added into the test cuvettes [24]. A volume of 50  $\mu$ L of respective platelet poor plasma was then added to the extract solution and mixed well and incubated for 5 minutes at 37°C in the incubation channels [24]. Pre-incubated PTT reagent (PTT Automate) was added and incubated for 180 seconds. The equipment's timer-linked pipette was used to deliver 50  $\mu$ L of 0.025M calcium chloride into the reaction to the reaction cuvettes [24]. Clot formation was detected automatically by the equipment and results printed. The average for the three test runs for each sample was calculated.

### Statistical analysis

Data was transferred to Microsoft Excel then exported to Minitab version 21 (MinitabLLC, Pennsylvania, USA). The data was subjected to descriptive statistics and expressed as Mean  $\pm$  SEM, analyzed using One-way ANOVA followed by Tukey's post-hoc test for pairwise comparison of means. The values of  $p \leq 0.05$  were considered significant. Results were presented using bar charts.

## RESULTS

### Methanol extraction yield

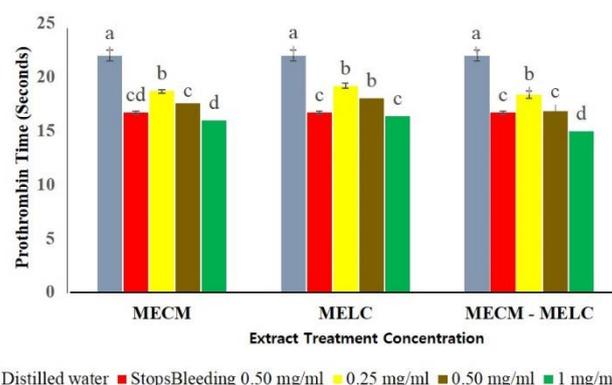
*C. megalocarpus* (H.) leaves yielded 17.3% of methanol extract. Leaves of *L. camara* (L.) produced 12% yield after extraction.

### Ex vivo effects of leaves extract on prothrombin time in normal mice plasma

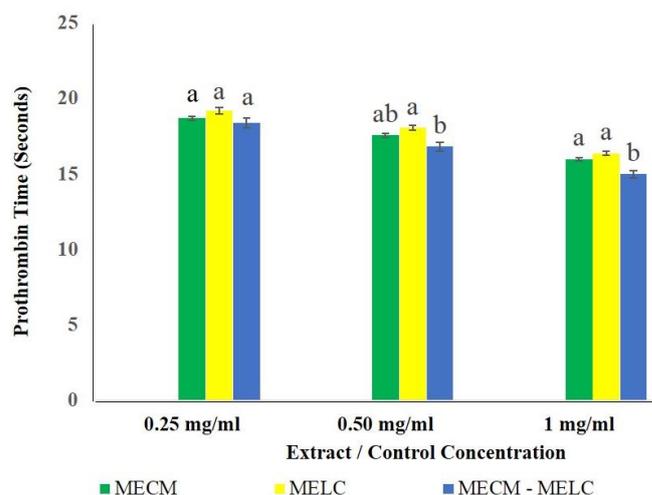
MECM and MELC extracts at 0.25, 0.5 and 1 mg/ml concentration reduced PT significantly ( $p < 0.05$ ) compared to the normal controls as shown in figure 1. At all the levels evaluated, extracts showed a dose dependent activity where higher concentration was found to show a lower PT ( $p < 0.05$ ).

The effects of MECM, MELC extracts and MECM-MELC blend were not significantly different ( $p = 0.112$ ) at 0.25 mg/ml concentration (Figure 2). The same trend was noted in the effects of MECM at the extract concentration of 0.5 mg/ml, compared to the blended leaves extract at a similar concentration, where the activity was not statistically different ( $p = 0.083$ ) a similar concentration. At 0.5 and 1 mg/ml concentration, the activity of MECM and MELC

extracts was not significantly different ( $p = 0.104$ ) compared to each other as illustrated in Figure 2.



**Figure 1.** Effects of leaves extracts on prothrombin time (PT). MECM (methanol leaves extract of *Croton megalocarpus*), MELC (methanol leaves extract of *Lantana camara*), MECM-MELC (methanol *Croton megalocarpus* and *Lantana camara* blended leaves extract). Bars with a different letter within individual clusters are significantly different (one-way ANOVA followed by Tukey post hoc test  $p < 0.05$ ). Letters in order of increasing hemostatic activity: a, b, c, d.



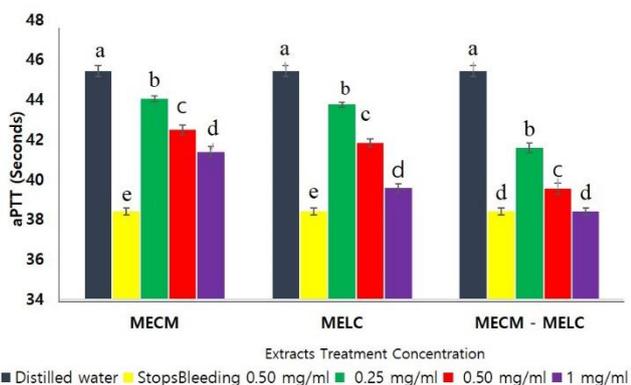
**Figure 2.** Comparison of effects leaves extracts on PT. MECM (methanol leaves extract of *Croton megalocarpus*), MELC (methanol leaves extract of *Lantana camara*), MECM-MELC (methanol *Croton megalocarpus* and *Lantana camara* blended leaves extract). Bars with a different letter within individual clusters are significantly different (one-way ANOVA followed by Tukey post hoc test;  $p < 0.05$ ). Letters in order of increasing hemostatic activity: a, b.

### Ex vivo effects of leaves extract on activated partial thromboplastin time in normal mice plasma

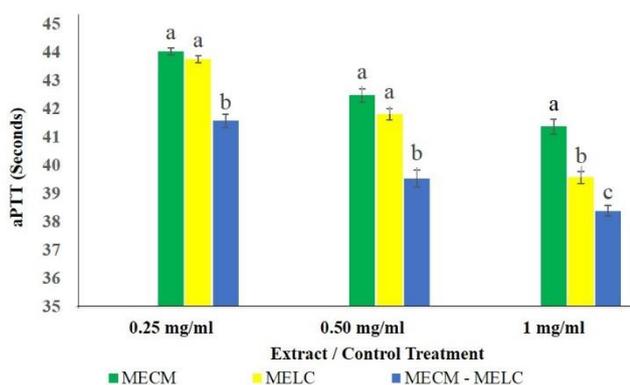
MECM, MELC extracts and the MECM - MELC blend caused a significant shortening of activated Partial Thromboplastin Time *ex vivo* compared to the negative control, as illustrated in Figure 3. The effects

of the extract on aPTT were dose dependent, where the highest concentration had a higher reduction in coagulation time ( $p < 0.05$ ; Figure 3).

The effect of the blend extract on aPTT was significantly higher ( $p < 0.05$ ) compared to separate extracts of the same plants across all concentrations studied as illustrated in Figure 4. At lower extract concentrations (0.25 and 0.5 mg/ml) the effects of MECM and MELC extracts on aPTT were, however, not significantly different ( $p > 0.092$ ). In contrast, at 1 mg/ml concentrations, the MELC extract had more activity than that of MECM ( $p < 0.05$ ; Figure 4).



**Figure 3.** Comparison of effects of leaves extracts on aPTT. MECM (methanol leaves extract of *Croton megalocarpus*), MELC (methanol leaves extract of *Lantana camara*), MECM-MELC (methanol *Croton megalocarpus* and *Lantana camara* blended leaves extract). Bars with a different letter within individual clusters are significantly different (one-way ANOVA followed by Tukey post hoc test  $p < 0.05$ ). Letters in order of increasing hemostatic activity: a, b, c, d.



**Figure 4.** Comparison of effects of leaves extracts on aPTT. MECM (methanol leaves extract of *Croton megalocarpus*), MELC (methanol leaves extract of *Lantana camara*), MECM-MELC (methanol *Croton megalocarpus* and *Lantana camara* blended leaves extract). Bars with different letter within individual clusters are significantly different (one-way ANOVA followed by Tukey post hoc test  $p < 0.05$ ). Letters in order of increasing hemostatic activity: a, b, c.

### Phytochemical analysis of leaves extract

Qualitative phytochemical analysis on MECM extract revealed the presence of tannins, phenols, steroids, flavonoids, cardiac glycosides, terpenoids, reducing sugars and carbohydrates. MELC extract was found to have saponins, tannins, phenols cardiac glycosides, terpenoids, reducing sugars and carbohydrates as listed in table 2.

**Table 2.** Phytochemical Screen of methanol extracts of *C. megalocarpus* (H.) and *L. camara* (L.)

Phytochemical/ macromolecule	MECM	MELC
Saponins	-	+
Tannins	+	+
Phenols	+	+
Steroids	+	-
Flavonoids	+	-
Cardiac glycosides	+	+
Anthraquinones	-	-
Terpenoids	+	+
Reducing sugars	+	+
Carbohydrates	+	+
Proteins	-	-

Phytochemicals present in the extracts are denoted by (+) sign; while those absent are denoted by (-) sign. MECM (methanol leaves extract of *Croton megalocarpus*), MELC (methanol leaves extract of *Lantana camara*).

### DISCUSSION

Overall, leaves extract of MECM and MELC and those of MECM - MELC blend demonstrated a significant reduction of the PT and aPTT of treated plasma. This is an indication of hemostatic activity involving extrinsic, intrinsic and common pathways of blood coagulation. The activity of the individual extracts of both plants on PT was noted to be the same at similar concentrations. However, MELC extract demonstrated higher activity on aPTT compared to MECM at 1 mg/ml concentration.

The effects of the plant extracts evaluated in this study could be attributed to phytochemicals detected in the qualitative screen. Literature identifies tannins, phenols, flavonoids and serine proteases as some of the phytochemicals linked to a reduction of PT and aPTT in treated plasma [25]. Additionally, saponins are associated with the shortening of plasma coagulation time involving aPTT [25]. Saponins isolated from *Paris forgesii* plant were associated with a reduction of prothrombin time in normal plasma [26].

The presence of saponins and phenols in plant extracts is associated with their reduction of PT in normal plasma [27, 28]. In study involving *Chromolaena ordata*, it was noted that flavonoids and tannins had blood clotting properties, leading to acceleration of plasma coagulation [29].

Astragalin, a flavonoid isolated from an extract of *Rosa chinensis* (J.) flowers, caused significant shortening of PT compared to the model group [30]. A similar finding was made in evaluation of kaempferol and phloridzin, (both flavonoids) isolated from *Malus pumila* Mill flower extract, where the compounds shortened normal plasma PT [31]. Gallic acid and Vanillin acid, (both phenols) isolated from *Sedum aizoon* (L.) leaves extracts, were associated with a reduction in plasma Prothrombin Time [27]. Phloretin (a phenol) isolated from *Malus pumila* Mill flowers extract reduced PT of treated plasma [31]. Phenols and flavonoids are also associated with the reduced coagulation time affecting the extrinsic pathway [32, 33].

Gallic acid and Vanillin acid (both phenols), isolated *Sedum aizoon* (L.) leaves extract were linked with reduction of plasma aPTT and Thrombin time [27]. On the other hand, phloretin, (a phenol) isolated, from *Malus pumila* M. flower extract was found to reduce aPTT of treated plasma significantly [31]. Serine glycoprotease isolated from *Cucumis savitus* L. fruit extract reduced aPTT of treated plasma significantly [36]. Furthermore, saponins are generally associated with reduced blood clotting time when applied topically [32]. In a study involving *Paris polyphylla* V., the reduction in aPTT in normal plasma was attributed to saponins present in the extract [37].

Likewise, tannins have been found to reduce aPTT as demonstrated by activities of *Mirabilis jalapa* and *Paris polyphylla* V. extracts, in normal rabbit plasma [37, 38]. Strong complexes formed when tannins are mixed with proteins are associated with the phytochemical's ability to shorten coagulation time [39]. In a study conducted by Yin *et al.* (2018), kaempferol and phloridzin, (both flavonoids) isolated from *Malus pumila* M. flower extract decreased aPTT of treated plasma significantly [31]. In addition, Chalcone (X), a flavonoid, separated from *Oxytropis falcate* extract reduced plasma re-calcification time significantly [40].

Saponins, detected in MELC extract are postulated to mediate the aggregation of thrombin and fibrinogen during hemostasis, a mechanism that leads to shorter PT and aPTT coagulation time [25]. Flavonoids (found

in MECM extract) shorten PT and aPTT coagulation time by participating in the direct conversion of factor X to Xa, bypassing the time-consuming cascade within the intrinsic and extrinsic pathways [43]. Tannins (revealed in both extracts) are postulated to reduce PT and aPTT of treated plasma by enhancing the function of fibrinogen in hemostasis by increasing its potency [42]. Phenols (found in both extracts) believed to shorten plasma coagulation time by facilitating the conversion of factor X to Xa in the common pathway, thereby reducing PT and aPTT [43].

The MECM-MELC blend extract had an enhanced activity lowering the PT and aPTT of mice plasma significantly at the highest evaluated concentration compared to individual extracts at similar concentrations. This is an indication of an additive synergistic effect, which could be linked to an increasing pool of active compounds and a possible interaction among hemostatic phytochemicals. In a study involving ethanolic flower extracts of *Bauhinia acuminata*, the presence of flavonoids, saponins and phenols created synergistic effects on hemostasis [41]. In a similar study, blend leaves extract of *Vernonia amygdalina* and *Chromolaena ordata* caused a significant reduction of PT in Wistar rat plasma [34]. In a study on synergistic effects of honey and *Nigella sativa* extract, there was enhanced activity when blended compared to the control. This is attributed to the combined pool of flavonoids, phenolic acids, enzymes and vitamins which are known to improve wound healing [35].

## CONCLUSION

The findings of this study indicate that leaf extracts of *C. megalocarpus* (H.) and *L. camara* (L) can be used to control peripheral bleeding. This study recommends that further study be conducted on the effects of fractions of methanol extracts of both plants on plasma coagulation, bleeding time and wound healing properties. *L. camara* (L.) and *C. megalocarpus* (H.) plants have also been identified as candidates for future drug development studies.

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## AUTHOR CONTRIBUTIONS

Hezron Muindi and Mathew Ngugi were involved in the conception and design of the experiments. Hezron Muindi, Cromwell Kibiti and Mathew Ngugi contributed to perform the experiments. Cromwell Kibiti and Mathew Ngugi analyzed data. Hezron Muindi contributed to drafting the article. Cromwell Kibiti and Mathew Ngugi contributed to revising it critically for important intellectual content. Mathew Ngugi made the final approval of the version to be published.

## CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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