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Review Article

Analyzing Calotropis gigantea's Potential as an Anticoagulant Human Fibrinogen: Proteolytic Activity on Latex

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ABSTRACT

Calotropis gigantea, known as “Sweta Arka,” is a perennial herb belonging to the Apocynaceae family, predominantly found in barren regions of India. This plant has been traditionally utilized for various ailments, exhibiting a range of pharmacological activities including analgesic, anti-inflammatory, and anti-diarrheal properties. Recent studies have highlighted its potential in treating cardiovascular diseases, specifically thrombus formation, which is critical in acute coronary syndromes and strokes. The plant's latex contains hydrolytic enzymes, notably proteases, which may influence hemostasis. This study investigates the proteolytic activity of C. Gigantea latex on human fibrinogen and its implications for coagulation and fibrinolysis. The extraction of active compounds from the leaves was performed using 70% ethanol and methanol, with subsequent analysis revealing the pharmacological properties of these extracts. These findings underscore the potential of C. Gigantea as a therapeutic agent in managing blood coagulation and related disorders.

INTRODUCTION

In Ayurvedic medicine, the names “Sweta Arka” and “Raktha Arka” refer to Calotropis gigantea and Calotropis procera, respectively. Calotropis gigantea R.Br., belonging to the Apocynaceae Family and the Asclepiadaceae. Subfamily, is a perennial herb that is primarily found in wastelands and desolate areas of India. Traditionally, a variety of common disorders are

treated using Calotropis gigantea alone or in conjunction with other herbal plants. The plant is said to possess analgesic (Pandian et al. 2013), anti-diarrheal (Rathod et al. 2009), and free radical scavenging (Chitme et al. 2004) properties. The plant's fresh juice (from young buds) is said to reduce inflammatory conditions and treat toothache and earache (Pathak and Argal. 2007, Mushir et al. 2016). It is said to lessen

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inflammatory illnesses as well (Adak and Gupta, 2006, Das et al. 2009). Additionally, the plant has been found to be used to treat anxiety, central nervous system diseases, and convulsions (Aragal and Pathak. 2006, Ghule et al. 2014, Khan et al. 2014, Lima et al. 2012). Additionally, the plant has been reported to have antimalarial (Satish et al. 2017, Kaushik et al. 2015, Kovendan et al. 2012) and anticancer (Habib and Karim. 2011, Bhat and Sharma. 2013, Mutiah et al. 2018) properties. It has also been used to treat fevers, elephantiasis (Chitme et al. 2005), rheumatism (Timilsina et al. 2020, Saratha and Subramanian. 2011, Patil et al. 2007), wound healing (Deshmukha et al. 2009).



Asthma (Mayee et al. 2011), diabetes (Rathod et al. 2011, Jatin et al. 2014, Manivannan and Shopna. 2017). Active Phytoconstituents in plant parts including triterpenoids (Gupta and Ali. 2000), flavonoids (Seniya et al. 2011, Kori and Alawa. 2014) glycosides and proteases (Rajagopalan et al. 2019) volatile long chain fatty acids (Singh et al. 2014) have been isolated from The various parts of the plant *Calotropis gigantea*. Cardiovascular disease and stroke are the major causes Of morbidity and mortality in high-income countries. Although many factors contribute to

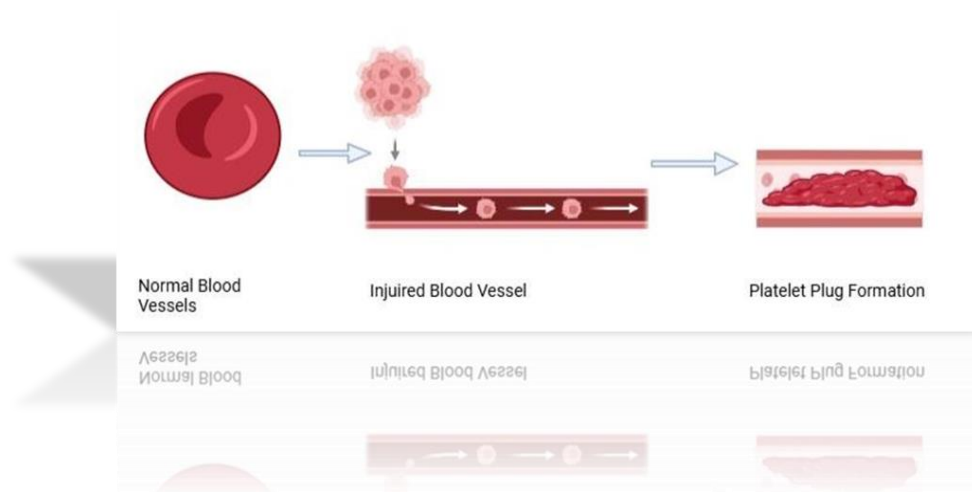
the development of Cardiovascular disease, thrombus formation is the main Trigger even Calfiontraocpui teigcaonrteoanary syndrome and stroke.(1) Herbs and plants have in the conventional medical system as a source of therapeutic chemicals Since ancient times, (2) *Calotropis* species, which are endemic to the tropical world and are members of the Asclepidaceae family of plants, are among the most well-known plants. To the regions of Asia and Africa that are tropical or subtropical(3) Several hydrolytic enzymes were found in the aqueous extract of *C. gigantea* latex (Abraham and Joshi, 1979; Senugupta et al., 1984). Proteases are found to be quite numerous among them and are most likely in charge of the latex's varied pharmacological characteristics. Numerous proteases from the venoms of snakes and mammals are known to obstruct Proteases are found to be quite numerous among them and are most likely in charge of the latex's varied pharmacological characteristics. A number of proteases found in the venoms of snakes and mammals have been shown to disrupt hemostasis by acting as either procoagulants or anticoagulants (Kornalik, 1990; Markland, 1991; Siigur & Siigur, 1992). This work describes the pharmacological characteristics of the proteolytic activity derived from the crude portion of *C. gigantea* latex in relation to the hydrolysis of human fibrinogen, which causes plasma to coagulate and the latex to subsequently exhibit plasmin-like activity. A mixture of cells (platelets, white blood cells, and red blood cells) and plasma make up blood, a crucial tissue. Blood flow must be smooth in order for the body to function physiologically. Still, the blood-clotting mechanism is equally significant. Blood clotting is a normal and essential process that helps to preserve hemostasis. [2] by producing



clumps or clots and helps to avoid death, even from slight injuries, by stopping bleeding from a damaged or injured vessel [1]. Thus, blood clotting plays a significant role in hemostasis under physiological settings [1], with platelet activity, coagulation factor cascade, clot inhibition/fibrinolysis, and the vessel wall serving as its main constituents. Together, they

cooperate to prevent protracted bleeding or thrombosis [3]. But a deficiency in this process can result in excessive bleeding or spontaneous bleeding, like hemophilia; on the other hand, a deficiency in blood clots can cause thrombosis and deadly embolism.

Blood clotting Process



METHOD PLANT MATERIAL

In December 2014, a *C. Gigantea* plant was harvested from its natural population that was growing in Vilad ghat, Ahmednagar, India. Standard keys and descriptions were used in the field to identify the plant material. After being cleaned to remove dirt and dust particles, the plant's leaves were allowed to air dry. In order to reduce or eliminate the possibility of losing active molecules, light exposure was avoided. Using a grinder, the air-dried leaves were ground into a powder and kept at room temperature until.

Equipment

Centrifuge, shaker incubator, freeze dryer, rotary evaporator, grinder, orbital shaker, and digital coagulation and analyzer.

Chemical and reagents

The subsequent substances and agents Citrated tubes (Greiner Bio-One), heparin (Sigma-Aldrich, Germany), methanol and ethanol (Merk), DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Germany), PT reagent (Hemostat thromboplastin-SI, Human, Germany), and aPTT reagent (Human, Germany) were utilized. As well as calcium chloride solution (Human, German, Numerous).

Extraction procedure



Extractions of methanol and ethanol A week was spent incubating ten grams of powdered plant leaves in an orbital shaker set at room temperature (120 rpm) with one gram of the leaves soaked in 70 ml of ethanol and another ten grams in 70 ml of methanol. Subsequently, the mixtures were centrifuged at 5000 rpm for 5 minutes. The supernatants were evaporated using a rotary evapo-Rator. 1% dimethyl sulfoxide (DMSO) was used to dissolve the extracted powder of the studied plant to final concentrations of 100, 50, 25mg/ml.

Hot and cool extraction

10 g of powdered plant leaves were soaked in 100 ml of boiling, sterilized distilled water at 100 °C for a hot extraction. The mixture was then incubated for a week at 37 °C in a shaking incubator. The mixes were then centrifuged at 5000 rpm for 5 minutes. The supernatants were dried with A freeze drier. The plant powder that was acquired and tested was dissolved to final concentrations of 100, 50, and 25 mg/ml in cooled, sterilized, and distilled water. For the extraction of cold water. The process is the same as for hot water extraction, except instead of soaking the plant powder in 100 ml of sterile distilled water at 20 °C, it was incubated at room temperature (25 °C) in an orbital shaker.

Blood sample preparation

Twenty healthy participants (18-52 years Old, 57-93 kg weight) of both genders (10 Males and 10 females) were asked to supply Blood samples. Both medicine use and smoking cessation are requirements for participants. In citrated tubes,

nine parts blood were combined with one part of a 3.2% sodium citrate solution to collect the blood samples. The samples Were treated as the following; each blood Sample was centrifuged at 3000 rpm for 15 Min to obtain the Platelets Poor Plasma (PPP) [22]. Two hours following blood collection, PT and aPTT assays were performed on all samples. A digital coagulation analyzer (Coa DATA 4004, Laber Biomedical Technologies, GerMany) was used to record the clotting time for both tests. Every coagulation assay was carried out twice. For the PT and aPTT tests, The assays for PT and aPTT were conducted using heparin (0.016 mg/ml) as a positive control. In parallel, sterilized distilled water and 1% DMSO were employed as the alcoholic and aqueous extracts' negative controls, respectively.

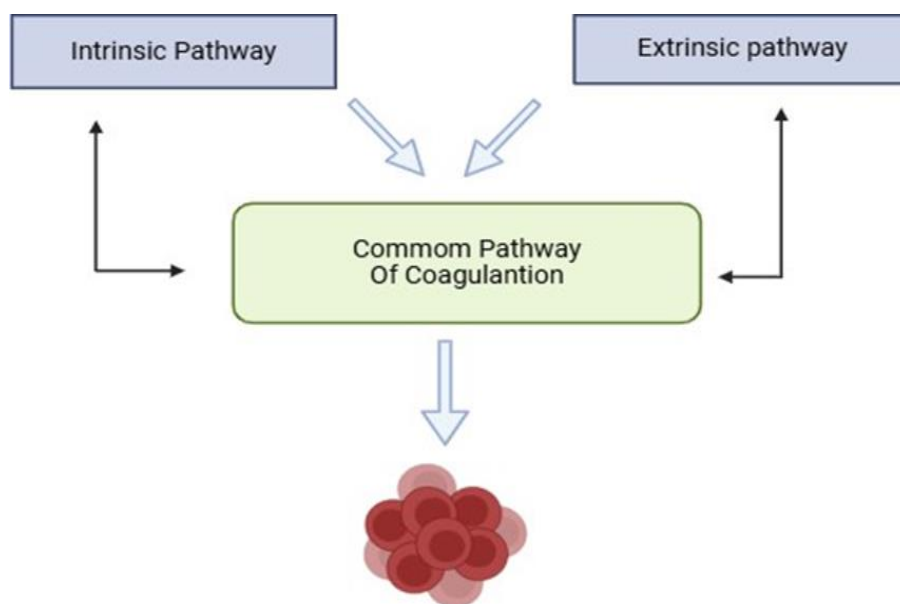
Prothrombin time (PT) assay

In vitro PT experiments involved incubating 50 µl of normal citrated blood sample (PPP) at 37°C for 5 minutes with 50 µl of each plant extract at varying concentrations (100, 50, and 25 mg/ml). Following the addition of 100 µl of PT reagent (Hemostat thromboplastin-SI. Human, Germany), the PT clotting time was promptly recorded.

The aPTT assay, or activated partial thromboplastin time

Invitro aPTT experiments involved incubating 50 µl of normal citrated blood sample (PPP) at varied concentrations (100, 50, and 25 mg/ml) for two minutes at 37°C with 50 µl of each plant extract. After that, 3 minutes were spent incubating 50 µl of aPTT reagent (Human, Germany).





Intrinsic Pathway Measured by partial thromplastin time.

Extrinsic Pathway Measured by Prothrombin time.

Test for International Normalized Ratio

An assessment of your blood clotting ability is provided by the International Normalized Ratio test. The steps involved in the INR test technique are: blood specimen A blood sample is obtained by a medical practitioner from a vein in your arm or from a drop of blood on your fingertip. Getting Ready To avoid infection, an alcohol swab is used to disinfect the area where the blood is extracted. The blood sample should be drawn prior to your daily dose of warfarin if you are taking it. Testing A laboratory receives the blood sample for examination. Outcomes The INR is a ratio that reveals how long it takes for your blood to clot. Your blood clotting time will be longer if your INR is higher.

Statistically analyze

t-test for independent samples in the StatistiCal software SPSS (Version 20) was used to statistically analyze the PT and aPTT mean Value values in order to ascertain whether there was a significant difference between the various examined plant extracts and the control. P-values less than 0.05 were regarded as significant. Dr. Ali Barakat of the Department of Statistics, Faculty of Science, An-Najah National University, carried out this statistical analysis.

RESULT

The anticoagulation results of the exam Ined hot water, cold water, ethanol, and methanol extracts of C. Procera showed noindividual variation among the examined Blood samples for both PT and aPTT ($P > 0.05$). Therefore, they could be considered A representative sample for this study. In addition, the negative controls (1% DMSO and Sterilized distilled H₂O) in the PT and aPTT Analysis in this study showed no significant effect relative to the normal control ($P > 0.05$).All evaluated extract types of C. Procera .At all



studied concentrations (100, 50, 25 Mg/ml) increased PT significantly ($P \leq 0.05$) .Relative to the normal control values. Moreover, compared with the positive heparin control, only the ethanol and methanol extracts at 100g/ml showed a similar

anticoagulant effect ($P > 0.05$), while other samples had less anticoagulant effect compared to heparin. Recorded data of the evaluated plant extract types at the different investigate.

Plant extracts	Concentration (mg/ml)	PT $\pm$ SD ^a (sec)	* P- value	** P- value
Hot water	100	77.17 $\pm$ 12.98	0.000	0.069

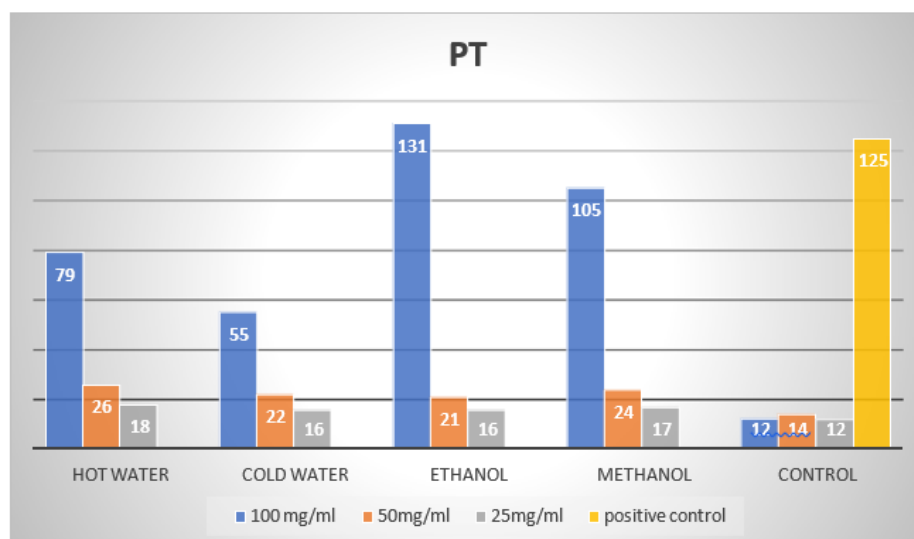
Table no. 1

	50	28.57 $\pm$ 7.50	0.000	0.001
	25	16.63 $\pm$ 1.78	0.000	0.000
Cold water	100	50.66 $\pm$ 6.66	0.000	0.007
	50	22.16 $\pm$ 1.98	0.000	0.000
	25	15.34 $\pm$ 0.97	0.000	0.000
Ethanol	100	128.52 $\pm$ 24.45	0.000	0.895
	50	27.20 $\pm$ 2.56	0.000	0.001
	25	16.19 $\pm$ 1.32	0.000	0.000
Methanol	100	105.99 $\pm$ 17.76	0.000	0.436
	50	26.41 $\pm$ 2.87	0.000	0.001
	25	16.02 $\pm$ 1.37	0.000	0.000
Normal control (blood sample with- out plant extract)		12.76 $\pm$ 0.78		
Negative control (sterilized distilled H ₂ O)		12.58 $\pm$ 0.83		
Negative control (1%DMSO)		12.35 $\pm$ 0.95		

d concentrations on PT are Represented in Table (1) and Graph exhibit the recorded data for the assessed plant extract kinds at the various studied concentrations on PT. P-value ≤ 0.05 is significant [between the different extract types investigated

concentrations relative to the Normal control]. **P-value ≤ 0.05 is significant [between the different extract types investigated concentrations relative to the Positive control (heparin= 0. 016 mg/mGl)].





Graph No.1

Standardation

The PT values of the investigated extracts of *Calotropis procera* at the various assessed concentrations (100, 50, and 25 mg/ml) are shown in Figure (1). However, every *Calotropis procera* distinct assessed extract type showed a substantial extended aPTT ($P \leq 0.05$) at 50 and 100 mg/ml doses. When compared to the

usual control (25.21 ± 1.75 seconds), the anticoagulation activity at a concentration of 100 mg/ml (420.00 ± 0.00 sec) is deemed remarkable. On the other hand, all examined extract kinds at a 25 mg/ml concentration demonstrated the opposite outcome, a decline in aPTT. Zero seconds ($P \leq 0.05$) up to a point where blood coagulation occurs instantly.

Plant extracts	Concentration (mg/ml)	aPTT $\pm$ SD ^a (sec)	* P- value	** P-value
Hot water	100	420.00 $\pm$ 0.00	0.000	0.85
	50	51.70 $\pm$ 27.06	0.000	0.000
	25	0.00 $\pm$ 0.00***	-----	-----
Cold water	100	420.00 $\pm$ 0.00	0.000	0.85
	50	129.97 $\pm$ 56.66	0.000	0.000
	25	0.00 $\pm$ 0.00***	-----	-----
Ethanol	100	420.00 $\pm$ 0.00	0.000	0.85
	50	86.70 $\pm$ 28.85	0.000	0.000
	25	0.00 $\pm$ 0.00***	-----	-----

Table No.2

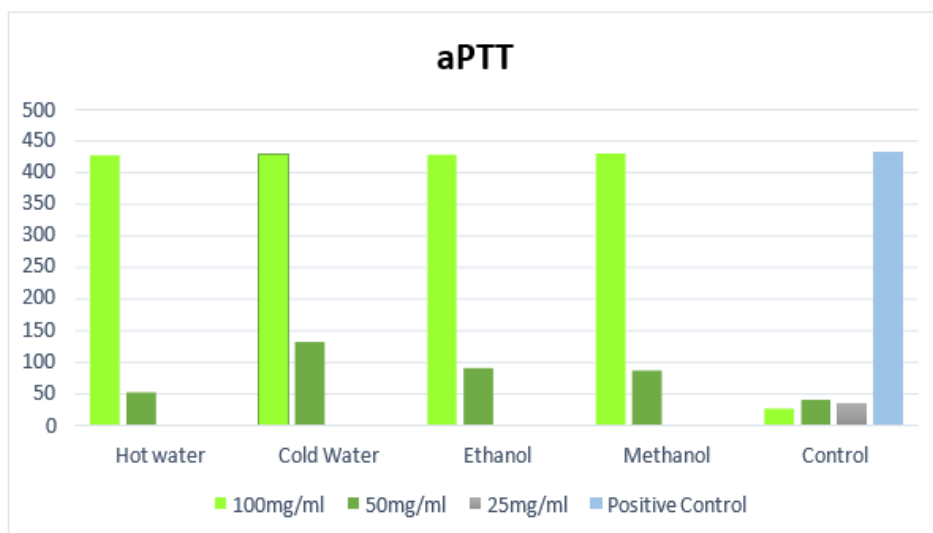
Methanol	100	420.00 $\pm$ 0.00	0.000	0.85
	50	79.97 $\pm$ 16.39	0.000	0.000
	25	0.00 $\pm$ 0.00***	-----	-----
Normal control (blood sample without plant extract)		25.21 $\pm$ 1.75		



Negative control (SDW)		34.55±3.89		
Negative control (1%DMSO)		29.20±2.66		
Positive control (Heparin = 0.016 mg/ml)		420.00±0.00		

Optionally, each assessed plant extract. Similar anticoagulation bioactivity was shown by types at 100 mg/ml concentrations on the examined blood samples aPTT in comparison to the positive heparin control ($P>0.05$). Consequently, they demonstrated the strongest anticoagulant effect in contrast to the concentrations of other plant extract (50 and 25 mg/ml). All assessed extract kinds' aPTT recorded results at all examined concentrations are shown in (Table 2 and Figure

2). The aPTT values of the investigated extracts of *Calotropis procera* at concentrations of 100, 50, and 25 mg/ml are shown in Table (2). *P-values [between the various extract types studied concentrations relative to the normal control] ≤ 0.05 indicate significance. **P-value ≤ 0.05 is significant [between the different extract types investigated concentrations relative to the Positive control (heparin = 0.016 mg/ml)].



Graph No.2

*** Coagulant effect of the plant extracts at that concentration, zero aPTT.

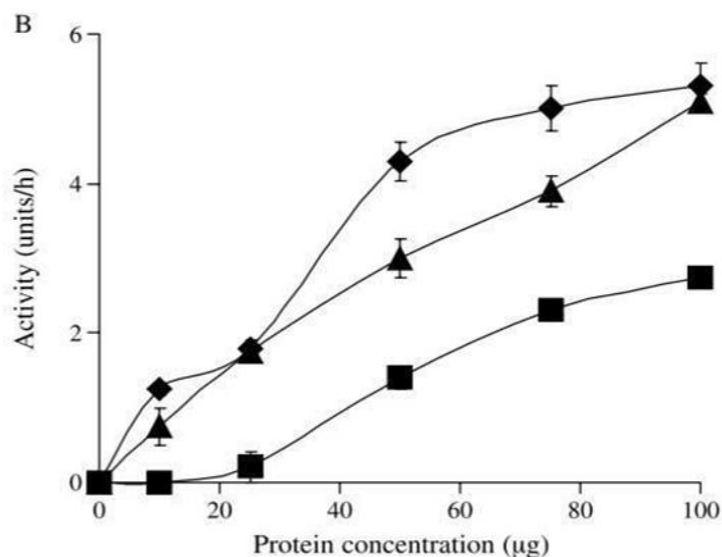
SDa stands for standard deviation.

The aPTT values of the investigated extracts of *Calotropis procera* at the various assessed

concentrations (100, 50, and 25 mg/ml) are shown in Figure (2). Additionally, all extract types at doses of 100 and 50 mg/ml significantly ($P\leq 0.05$) enhanced both PT and aPTT.

DISCUSSION





For varying durations (0–1 h), 50 mg of human fibrinogen and one microgram of crude *C. Gigantea* latex protein were incubated at 37 °C with 10 mM Tris–HCl buffer (pH 7.6) present. Visualizing fibrinogen breakdown products was done using SDS-PAGE (7.5%). A: 0 hours, B: 5, C: 10, D: 15, E: 30, F: 60 minutes of incubation. (C) Particular protease inhibitors decrease the human fibrinogenolytic activity of *C. Gigantea* latex. Protease-specific pre-incubation was performed on one microgram of *C. Gigantea* latex. After adding 50 mg of fibrinogen, the reaction was allowed to run for 15 minutes in the presence of a 10 mM Tris–HCl buffer (pH 7.6). The combination before incubation. The response was stopped after 30 minutes. Denaturing buffer is added. SDS-PAGE (7.5%) was used to visualize the prevention of fibrinogen breakdown. A: Fifty Micrograms of fibrinogen, B: 50 milligrams of fibrinogen C: 50 mg of fibrinogen, C: 1 mg of latex extract C: 50 mg fibrinogen, D: 100 mM IAA, and C: 1 mg latex extract C1 milligrams of latex extract C5 mM PMSF, E: 50 milligrams of fibrinogen C1 milligrams of latex extract EDTA at

5 mM and fibrinogen at 50 mg C5 mM EGTA, C1 mg latex extract G: fifty milligrams of fibrinogen C5 mM 1–10 phenanthroline; C1 mg latex extract Hydrolysis of blood clot by crude *C. Gigantea* latex, papain, and trypsin: a dose-dependent analysis. 0.5 ml of reaction mixture A cleaned blood clot was placed within a Tris–HCl buffer (10 mM; pH 7.6) and treated for two hours at 37 °C with varying concentrations of *C. Gigantea* latex (%), papain (&), and trypsin (), ranging from 10 to 100 mg of protein. The values show the mean GSEM (nZ5). (B) Hydrolysis of the plasma clot by crude *C. Gigantea* latex, papain, and trypsin in a dose-dependent manner. Mixture of reactions 0.5 milliliters included A plasma clot that has been washed was treated for two hours at 37 degrees Celsius with varying concentrations of crude *C. Gigantea* latex (%), papain (&), and trypsin (ranging from 10 to 100 mg of protein in the presence of Tris–HCl buffer (10 mM; pH 7.6). The values show the mean GSEM (nZ5). Hazardous and has negative impacts. It is well recognized that mammalian metalloproteinases are essential for atherogenesis, ovulation,



embryogenesis, intima proliferation, and angiogenesis (Lijnen, 2002). On the other hand, both intrinsic and extrinsic blood coagulation pathways involve serine proteases (Davie et al., 1979). Additional unique proteolytic sets. Enzymes from the latex of various plants were identified. They fall within the category of cysteine proteinases. Nevertheless, it is unclear how this class of proteases functions pharmacologically. According to Ashwani (1999), *C. Gigantea* latex is applied to wounds to both halt bleeding and promote healing. These pharmacological effects are indirectly linked to the latex's proteolytic enzymes. Although a number of cysteine proteases have been identified and extracted from *C. Gigantea* latex, their exact pharmacological functions are still unknown (Abraham and Joshi, 1979; Senugupta et al., 1984). Casein and other substrates, including human fibrinogen and fibrin contained in the plasma clot, are hydrolyzed by the crude latex extract. Aa chain, one of the fibrinogen components, was observed to breakdown preferentially despite having no discernible carbs. The Bb chain, on the other hand, broke down more slowly than the preceding one since it included carbohydrates. Table 1 shows how different inhibitors inhibit the hemorrhagic and proteolytic activities on different substrates. A percentage is used to express inhibition. The crude *C. Gigantea* latex protein was pre-incubated with the different inhibitors at the stated concentrations. Proteolytic and hemorrhagic activities were assessed in accordance with Section 2's instructions. IAA inhibits the hemorrhagic activity of crude *C. Gigantea* latex protein. Crude latex protein was injected at the back of the skin in two groups of mice, one with and one without IAA. Mice were put to sleep and slaughtered after three hours of

incubation. The skin's dorsal surface was removed, and the amount of bleeding on the inner surface was measured. The control group received an injection of saline. (A) Pre-incubated with 100 mM IAA, (B) 75 mg latex protein, and (C) 75 mg latex protein. Numerous venom proteases are extremely harmful and can cause bleeding in number of critical organs, among other adverse effects. Even at lesser concentrations, the crude extract of *C. Gigantea* latex strongly hydrolyzes every fibrinogen subunit and crude fibrin clot. Nevertheless, the crude extract causes bleeding at the injection site at higher doses. The procoagulant activity that was reported. Furthermore, IAA totally inhibited haemorrhagic activity, suggesting a potential direct participation of cysteine protease(s). The latex's ability to dissolve blood clots was far stronger than that of trypsin and papain enzymes, because wound healing requires the dissolution of blood clots. Such reactions are caused by in vivo plasmin enzymes in the presence of matrix metalloproteases (Lijnen, 2002). Thus procoagulant activity and clot dissolving property are probably responsible for the reported Pharmacological activities of the latex. These clot-inducing and clot-dissolving enzymes may function in a sequential manner in vivo and aid in the early coagulation mechanism-based wound healing phase. These results strongly imply that cysteine protease(s) are prevalent in crude latex and play a therapeutic role in wound healing and hemorrhage control.

CONCLUSION

The anticoagulant properties of *Calotropis* leaves are noteworthy, indicating their potential as a natural source for the development of medicinal medicines against blood coagulation disorders.



These effects could be attributed to the existence of bioactive substances in the leaves, which suggests that more study may be necessary to identify and isolate these molecules for use in medicine. All things considered, calotropis leaves offer a potential way to investigate substitute anticoagulant treatments; nonetheless, more research is required to completely comprehend their mechanisms and safety profiles. It is clear from this investigation that *C. Gigantea* extracts, both aqueous and ethanolic, have anticoagulant qualities. Nevertheless, *C. Gigantea*'s *in vivo* activity has not yet been identified and is being investigated. Based on the aforementioned findings, it can be inferred that society may be able to use the *C. Gigantea* component to treat cardiovascular conditions.

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