

## Evaluation of anticoagulant activity of aqueous extract of *Cestrum nocturnum*

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### Abstract

The study evaluated anticoagulant properties of the aqueous extract of *Cestrum nocturnum* using a PTT-Activated Partial Thromboplastin Time, PT- Prothrombin Time & TT-Thrombin Time as standard procedures. For *in vitro* coagulation assays, aqueous extract of plant prolonged APTT, TT, and PT clotting times in a dose-dependent manner (Table 7). It prolonged APTT clotting time from  $45 \pm 2$  (2mg/mL) to  $82.2 \pm 2.63s$  (10mg/mL), PT clotting time from  $20.4 \pm 1.49$  (2mg/mL) to  $31.4 \pm 2.15s$  (10mg/mL), and TT clotting time from  $9.2 \pm 1.16$  (2mg/mL) to  $17.4 \pm 1.01s$  (10mg/mL) at the concentration of 2 to 10mg/mL. Heparin prolonged APTT and PT clotting times more than 111.8s and 40.8s, respectively, at a concentration of 1 IU/mL. Heparin prolonged TT clotting times more than 20.6s at a concentration of 1 IU/mL.

The phytochemical screening of the plant confirm the presence of saponin in the water and ethanolic extract, Alkaloid in all the extract except hexane extract, tannin in water, ethanol and methanolic extract, amino acid in water and ethanolic extract, carbohydrate in water and methanolic extract and triterpenoids and glycoside were absent in all the extracts. The results demonstrated that the aqueous extract of *Cestrum nocturnum* possesses pharmacologically active anticoagulant principles that could be isolated and evaluated for clinical or physiological purposes.

**Key Words:** Anticoagulant, Thromboplastin Time, Prothrombin, *Cestrum nocturnum* etc

### Introduction

Medicinal plants have been used as a source of medicine in all cultures during the last decades. It is gradually becoming popular throughout the world [1]. Secondary metabolites of plants play an important role in medical care for a good percentage of world population and have been the source of inspiration for several major pharmaceutical drugs. Around 100 plant species have contributed significantly to modern drugs. Approximately half of the world's 25 best selling pharmaceutical agents are derived from natural products [2]. Thus, emphasis is now given on the standardization of herbal medicines by screening of biological activities of medicinal plants and isolation active principles from them.

During the last decade, the use of traditional medicine has expanded globally and is gaining popularity. It has continued to be used not only for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system [3]. According to WHO, herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries [4].

Since the beginning of civilization, plants have been used to treat infections and diseases. A medicinal plant is any plant that has medical effects or medical properties. The chemical components present in plants have medicinal values which produce definite physiological actions in the body. These components are called phytochemicals, the most important of these bioactive groups being: alkaloids, steroids, terpenoids, flavonoids, tannins and phenolic compounds [5]. Herbal remedies prepared from garlic (*Allium sativum*) are believed to inhibit platelet activation [5,6]. Tomatoes (*Lycopersicon esculentum*) may protect against cardiovascular diseases by inhibiting platelet aggregation [7]. *Melicope semecarpifolia* has shown antiplatelet aggregation properties [8]. *Melilotus albus* contains the chemical coumarin that exhibits anti-clotting activities [9]. Few other plant species that provide medicinal value have been scientifically evaluated for their possible medical application. *Ocimum basilicum* and *Petroselinum crispum*, are among other plants being used for their haemostatic and cardiovascular effects [10,11]. *Panax notoginseng* and *Panax quinque folium* are good sources of lead compounds for novel anti-platelet and anti-coagulant therapeutics [12]. Plants species that have been used to treat blood diseases including arterial hypertension are *Arbutus unedo* (Ericaceae) and *Urtica dioica* (Urticaceae), [13]. Other anti-clotting medicinal plants

include angelic root, anise, borage, devils claw, papain, ginseng, ginkgo, horse chestnut, alfalfa, red clover, fever few, passionflower herb and garlic [14]. Interestingly, some natural compounds in the diet may inhibit platelet activation [11].

Therefore, the search on new drugs must be continued and natural products from plants, microorganisms, fungi and animals can be the source of innovative and powerful therapeutic agents for newer, safer and affordable medicines [15,16].

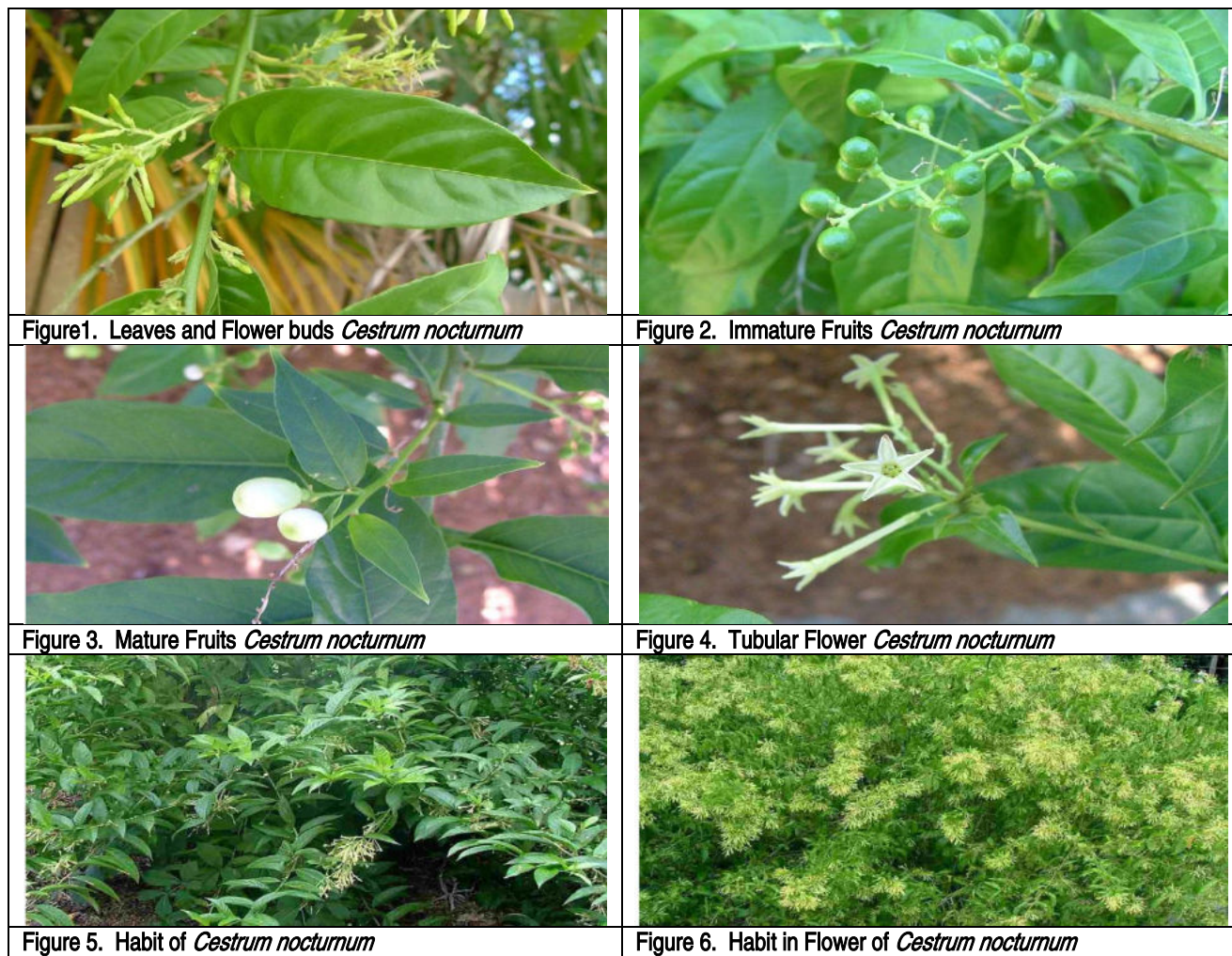
### Plant Profile

### Scientific Name

*Cestrum nocturnum* L.

### Scientific classification

Kingdom: Plantae  
 (unranked): Angiosperms  
 (unranked): Eudicots  
 (unranked): Asterids  
 Order: Solanales  
 Family: Solanaceae  
 Genus: *Cestrum*  
 Species: *C. nocturnum*



Useful Parts: Root, bark, leaves, flowers, and seeds. It is an ornamental plant. Flowers are used in perfume industry.

### Chemical Constituents

Phytochemical screening of methanol extract of stems yielded flavonoids, saponins, tannins, triterpenes, and carbohydrates. Phytochemical screening of aerial parts showed saponins, triterpenes, sterols, flavonoids, tannins, and Coumarins. [17]. Phytochemical analysis of various extracts yielded carbohydrates,

proteins, flavonoids, tannins, cardiac glycosides, saponins, and alkaloids. [18]

## Material & Method

### Plant Material

#### Collection and authentication of *Cestrum nocturnum*

The plant materials of *Cestrum nocturnum* was collected in and around Sri SatyaSai University of technology and Medical Sciences. Sehore, India in the month of March 2015. Identity of the material was confirmed by the NISCAIR, New Delhi with voucher specimens deposited at the Herbarium and also in the Sri SatyaSai University of technology and Medical Sciences. Sehore, India.

#### Extraction of *Cestrum nocturnum*

##### Successive solvent extraction

10g the air-dried powdered of *Cestrum nocturnum*, was successively extracted with the following solvents of increasing polarity in a soxhlet apparatus.

Hexane

Choloroform

Ethanol

Methanol

Water

All the extracts were concentrated by distilling the solvents and the extracts were dried in an oven. Each time before extracting with the next solvent, the marc was dried in an air. The marc was finally macerated with water for 24h to obtain the aqueous extract. The completion of the extraction was confirmed by evaporating a few drops of extract from the thimble on watch glass to observe that no residue remained after evaporation of the solvent. The liquid extracts obtained with different solvents were collected. The consistency, color, appearance of the extracts and their percentage yield were noted [19].

$$\% \text{ Yield} = \frac{\text{Weight of extract (g)}}{\text{Weight of dry powder(g)}} \times 100$$

##### Solvent removal

The next step of following solvent extraction was to separate the yield from the extractant which was performed by evaporation using a rotary evaporator followed by freeze drying. The earlier step is aimed at reducing the amount of solvent from the extract, which was performed under reduced pressure using IKA RV 10 digital rotary evaporator system (IKA Werke GmbH & Co, Germany) at a temperature below 40°C to minimize the degradation of thermolabile components (Seidel, 2006). Finally, all extracts were freeze-dried to remove all residual organic and aqueous mixtures

from the extracts and yield a completely-dry crude extracts. Dried extracts were stored in an airtight container at -20°C before use.

### Phytochemical analysis

A stock concentration of 1 % (W/ V) of each successive extract obtained using chloroform, hexane, ethanol, methanol and water was prepared using the respective solvent. These extracts along with positive and negative controls were tested for the presence of active phytochemicals viz: tannins, alkaloids, phytosterols, triterpenoids, falvonoids, cardiac glycosides, saponins, carbohydrates, proteins and amino acids etc following standard methods [19] as briefed below.

#### Tannins

**Ferric chloride Test:** Added a few drops of 5% ferric chloride solution to 2 ml of the test solution. Formation of blue color indicated the presence of hydrolysable tannins.

**Gelatin Test:** Added five drops of 1% gelatin containing 10% sodium chloride to 1 ml of the test solution. Formation of white precipitates confirmed the test.

#### Alkaloids

Approximately 50 mg of extract was dissolved in 5 ml of distilled water. Further 2M hydrochloric acid was added until an acid reaction occurred and filtered. The filtrate was tested for the presence of alkaloids as detailed below

**Wagner Test:** Two drops of Wagner's reagent was added to 1ml of the test solution along the the side of the test tube. The formation of yellow or brown precipitate confirmed the test as positive for alkaloids.

#### Phytosterols

**Liebermann-Burchard's Test:** The extract (2 mg) was dissolved in 2 ml of acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulfuric acid was added along the side of the test tube. A brown ring formation at the junction and the turning of the upper layer to dark green color confirmed the test for the presence of phytosterols.

#### Triterpenoids

**Salkowski Test:** Approximately 2 mg of dry extract was shaken with 1 ml of chloroform and a few drops of concentrated sulfuric acid were added along the side of the test tube. A red brown color formed at the interface indicated the test as positive for triterpenoids.



## Flavonoids

Shinoda test: A few magnesium turnings and 5 drops of concentrated hydrochloric acid was added drop wise to 1 ml of test solution. A pink, scarlet, crimson red or occasionally green to blue color appeared after few minutes confirmed the test.

## Saponins

Foam Test: 5 ml of the test solution taken in a test tube was shaken well for five minutes. Formation of stable foam confirmed the test.

## Cardiac glycosides

Keller -Killiani test: Added 0.4 ml of glacial acetic acid and a few drops of 5% ferric chloride solution to a little of dry extract. Further 0.5 ml of concentrated sulfuric acid was added along the side of the test tube carefully. The formation of blue colour in acetic acid layer confirmed the test.

## Test for carbohydrates

Molisch's test: To 1 ml of test solution added a few drops of 1 % alpha-naphthol and 2-3 ml concentrated sulfuric acid along the side of test tube. The reddish violet or purple ring formed at the junction of two liquids confirmed the test.

## Test for amino acids

Millon's test: Added 5 drops of millons reagent to 1 ml of test solution and heated on a water bath for 10 min, cooled and added 1% sodium nitrite solution. Appearance of red color confirmed the test.

## Anti Coagulant Activity

The action of water extract on haemostatic system was assessed by the evaluation of its anticoagulant activity in prothrombin time (PT), activated partial thromboplastin time (aPTT) tests and thrombin time (TT) [20].

## Prothrombin Time (PT) test

The action in extrinsic pathway was evaluated by PT test, as previously described in literature, with a few modifications. The test was carried out using commercial reagent kits. Plasma (90  $\mu$ L) was mixed with 10  $\mu$ L of samples (0.1 – 2  $\mu$ g/ $\mu$ L) and incubated at 37 C

for 5 min. Then, 200  $\mu$ L of PT assay reagent (rabbit brain extract and calcium chloride) pre-warmed at 37 C for 10 min was added and the clotting time was recorded by a digital coagulometer (Model No-ABC2202 "Bioline Technologies, Thane, India). Plasma alone (only with vehicle) was used as control (absence of anticoagulant activity). Heparin (1 IU/mL) (Rinhepa®, United Biotech Pvt. Ltd., India) was used as positive control.

## Activated partial Thromboplastin time (aPTT) test

The action in intrinsic and common pathways was evaluated by aPTT test, as previously described in literature, with a few modifications. The test was carried out using commercial reagent kits. Plasma (90  $\mu$ L) was mixed with 10  $\mu$ L of samples (0.1 – 2  $\mu$ g/ $\mu$ L) and incubated at 37 C for 5 min, before the addition of pre-warmed aPTT reagent (rabbit brain extract and ellagic acid) and incubation at 37 C for 2 min. Pre-warmed (37 C) 25 mM calcium chloride was then added and the clotting time recorded by a digital coagulometer (Model No-ABC2202 "Bioline Technologies, Thane, India). Plasma alone (only with vehicle) was used as control (absence of anticoagulant activity). Heparin (1 IU/mL) (Rinhepa®, United Biotech Pvt. Ltd., India) was used as positive control.

## Thrombin-induced clotting time assay (TT)

This assay measures the prolongation of thrombin generation. When human plasma is incubated with a compound which inhibits blood coagulation, the time taken for clot formation will be prolonged compared to the control (test devoid of inhibitor). In this assay, 200  $\mu$ L of human plasma (pre-incubated at 37 C for 5 min before use) was incubated with different concentrations of the extract for 5 min at 37 C; buffer and normal plasma served as the controls. Concentrations (1 IU/mL) of heparin were used as the reference anticoagulant. A fixed concentration (100  $\mu$ L) of bovine thrombin (2.5 U/ml, Sigma) was added to each sample to initiate reaction. The time for clot formation was recorded accordingly. Results were expressed as a prolongation time relative to controls.

## Statistical Analysis

All experimental values reflect an average of a minimum of 5 experiments. Error bars indicate standard deviation (SD) unless otherwise specified. Statistical significance was evaluated by the paired Student *t*-test. Values of *p* 0.05(\*) or *p* 0.01(\*\*) or *p* 0.001(\*\*\*) were considered to be statistically significant.

## Result

**Table 1.** The yield and colour of the extracts of *Cestrum nocturnum* obtained following extraction with different solvents in succession.

Extract	Extract yield (w/w)	Extract description
Water	18.7%	Brown, dry flake consistency
Methanol	5.2%	Dark brown, thick consistency
Ethanol	4.7%	Dark green, thick consistency
Hexane	0.8%	Light green, viscous oil consistency
Chloroform	1.3%	Dark green, flakes and oil-like consistency

The practical yield of extraction is tabulated in table no. 1, and from the data it can be observed that the among all the solvent used i.e. water, methanol, ethanol, hexane and chloroform; water extract shows 18.7% yield, methanol extract shows 5.2% yield, ethanol extract shows 4.7% yield, hexane extract shows 0.8% and chloroform extract shows 1.3% yield. Now depending upon the yield it was observed that the maximum practical yield was obtained from the water extract. So for the further study the water extract chosen.

**Table 2.** Phytochemical screening of *Cestrum nocturnum* extracts

	Water extract	Methanol extract	Ethanol extract	Hexane extract	Chloroform extract
<b>Saponin</b>					
Foam test	+	-	+	-	-
<b>Alkaloid</b>					
Wagner's test	+	+	+	-	+
<b>Flavonoid</b>					
Shinoda test	+	+	-	-	-
<b>Tannin</b>					
Gelatine test	+	+	-	-	-
Ferric chloride Test	+	-	+	-	-
<b>Amino Acid</b>					
Millon's test	+	-	+	-	-
<b>Carbohydrate</b>					
Molisch's test	+	+	-	-	-
<b>Phytosterol/Triterpenoids</b>					
Liebermann-Burchard's test	-	-	-	-	-
Salkawaski's test	-	-	-	-	-
<b>Glycoside</b>					
Keller Killiani's test	-	-	-	-	-

The phytochemical screening of the plant confirm the presence of saponin in the water and ethanolic extract, Alkaloid in all the extract except hexane extract, tannin in water, ethanol and methanol

extract, amino acid in water and ethanolic extract, carbohydrate in water nadmethanolic extract and triterpenoids and glycoside were absent in all the extracts.

**Table 3.** Anticoagulant activity of aqueous extract of *Cestrum nocturnum*

Sample	Dose	aPTT (seconds)	PT (seconds)	TT (seconds)
Control	Saline	34.4 ± 2.87	15 ± 2	7.4±1.01
Heparin	1 IU/mL	111.8 ± 4.79	40.8 ± 2.48	20.6 ± 2.15
Aqueous extract	2 mg/ml	45 ± 2	20.4 ± 1.49	9.2 ± 1.16
Aqueous extract	4 mg/ml	63.8 ± 2.71	22.4 ± 2.41	11.8 ± 1.16
Aqueous extract	6 mg/ml	75 ± 3.16	26.6 ± 2.15	13.8 ± 1.6
Aqueous extract	8 mg/ml	80.6 ± 2.15	29.4 ± 1.01	14.6 ± 1.95
Aqueous extract	10 mg/ml	82.2 ± 2.63*	31.4 ± 2.15*	17.4 ± 1.01*

Values are expressed as mean ± SD. p 0.05(\*) compared to control (n=5)

aPTT-Activated Partial Thromboplastin Time  
 PT- Prothrombin Time  
 TT-Thrombin Time

The aqueous extract of *Cestrum nocturnum* evaluated for *in vitro* anticoagulant activity at the concentration of 2mg/mL to 10mg/mL

and results are furnished in the table no. 3 which shows the Activated Partial Thromboplastin Time(aPTT), Prothrombin Time(PT) &Thrombin Time (TT)approximately dose dependent result and on comparing with the standard Control the dose of 10mg/mL was found to be significant.

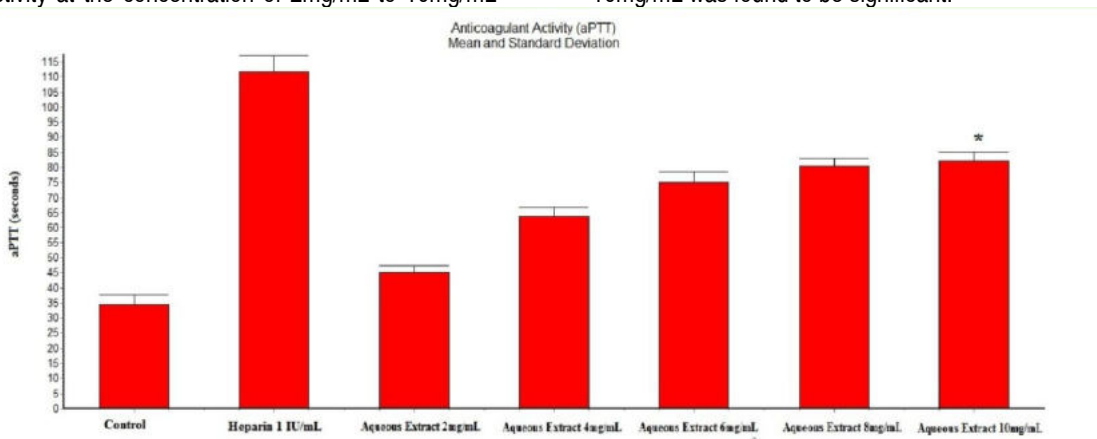


Figure 7. Comparison of effect of different concentrations of the test sample and the standard on *in vitro* Anticoagulant activity (aPTT)

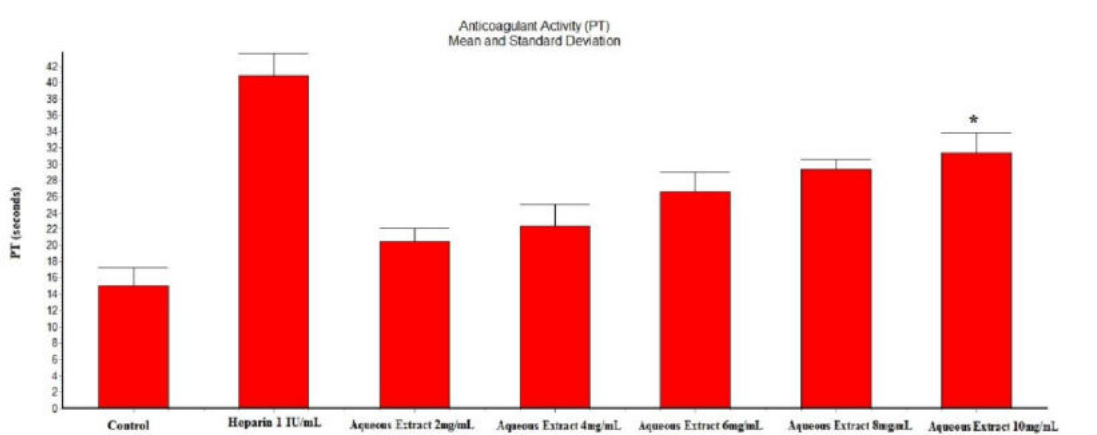


Figure 8. Comparison of effect of different concentrations of the test sample and the standard on *in vitro* Anticoagulant activity (PT)

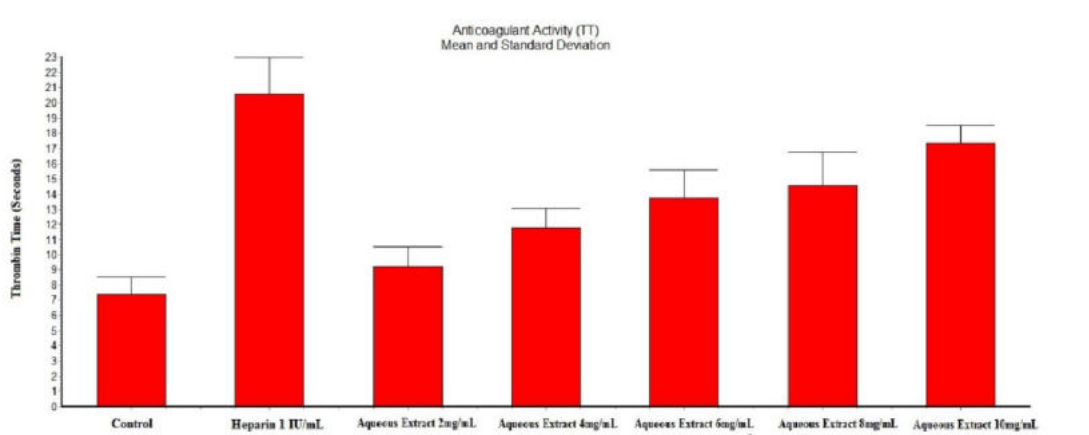


Figure 9. Comparison of effect of different concentrations of the test sample and the standard on *in vitro* Anticoagulant activity (TT)

## Discussion

The anticoagulant activities of AECN were measured by APTT, PT, and TT. APTT is used to evaluate the coagulation factors such as VIII, IX, XI, XII, and prekallikrein in intrinsic coagulation pathway while PT is used to evaluate the coagulation factors V, VII, and X in extrinsic coagulation pathway (Ebbeling et al, 1992). TT reflects the blood coagulation status that transforms fibrinogen into fibrin, which is directly induced by the addition of thrombin. The test only detects disturbances in the final stages of coagulation, especially dysfibrinogenemia or the presence of thrombin inhibitors (Blair and Flaumenhaft 2009). In our study, the results of APTT, PT, and TT assays *in vitro* showed that AECN significantly prolonged APTT, PT, and TT clotting times in a dose-dependent manner.

For *in vitro* coagulation assays, aqueous extract of plant prolonged APTT, TT, and PT clotting times in a dose-dependent manner (Table 7). It prolonged APTT clotting time from  $45 \pm 2$  (2mg/mL) to  $82.2 \pm 2.63s$  (10mg/mL), PT clotting time from  $20.4 \pm 1.49$  (2mg/mL) to  $31.4 \pm 2.15s$  (10mg/mL), and TT clotting time from  $9.2 \pm 1.16$  (2mg/mL) to  $17.4 \pm 1.01s$  (10mg/mL) at the concentration of

2 to 10mg/mL. Heparin prolonged APTT and PT clotting times more than 111.8s and 40.8s, respectively, at a concentration of 1 IU/mL. Heparin prolonged TT clotting times more than 20.6s at a concentration of 1 IU/mL.

## Conclusion

From all the result found during evaluation of *in vitro* anticoagulant activity of aqueous extract of *Cestrum nocturnum*, dose dependent anticoagulant activity may mainly exhibit anticoagulant activity correlating with the intrinsic coagulation process. Further work has to been done for isolation of active phytochemical constituents from the plant.

## Conflicts of interests

The authors declare no conflicts of interests.

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