International Journal of Phytomedicine 8 (2016) 491-499
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Isolation, evaluation and characterization of isolated compounds from aqueous extract of *Cestrum nocturnum*

Chandra Kishore Tyagi[1]*, Deenanath Jhade[1], Sunil Kumar Shah[1]

***Corresponding author:**

Chandra Kishore Tyagi

[1]College of Pharmacy, Sri Satya Sai University of Technology and Medical Sciences, Sehore, Madhya Pradesh, India

Abstract

The aim of present study was to isolate, characeterise and evaluation anticoagulant properties of the isolated compound from aqueous extract of *Cestrum nocturnum* using aPTT-Activated Partial Thromboplastin Time, PT- Prothrombin Time & TT-Thrombin Time as standard procedures. From all the result it can be concluded that water extract of *Cestrum nocturnum* plant was found to be best among all the extract under study and from the isolates of water extract of *Cestrum nocturnum* plant AE5 was found to be best activity (anti-platelet, Fibrinolytic and anti-coagulant). Further fractionation of water extract generated two isolates AE5a & AE6a and after their pharmacological activity they were found to be potent phytochemical constituents. From the structural elucidation study of AE5a & AE6a were identified as Rutin and quercetin respectively. From this study it was concluded that the flavoinoidal compound i.e. Rutin and Quercetin were present in the *Cestrum nocturnum* plant and they both responsible for the anti-platelet, Fibrinolytic and anticoagulamt activity of plant.

Key Word: *Cestrum nocturnum*, Thromboplastin, Prothrombin, Rutin, Quercetin.

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 worlds 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 sativam)* are believed to inhibit platelet activation [5,6]. Tomatoes (*Lycopersicum esculentum*) may protect against cardiovascular diseases by inhibiting platelet aggregation [7]. *Melicope semecarpifolia* has shown antiplatelet aggregation properties [8]. *Melilotusalbus* 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 *Petrol selinumcrispim,* are among other plants being used for their haemostatic and cardiovascular effects [10,11]. *Panaxnoto ginseng* and *Panaxquinque 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, gingko, 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

DOI:10.5138/09750185.1971

the source of innovative and powerful therapeutic agents for newer, safer and affordable medicines [15,16].

Material and Methods

Collection and authentication of plant material

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 Satya Sai University of technology and Medical Sciences. Sehore, India.

Preparation of Extract

Successive solvent extraction [17]

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.

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

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.

Preliminary Phytochemical Studies

Preliminary phytochemical tests were performed for chemical constituents such as alkaloids, carbohydrates, glycosides, steroids, tannins, proteins and amino acids, fixed oils and fats, flavonoids and saponins and it was found that flavonoids were present.

Chromatographic techniques

Column Chromatography

Silica gel (70-230 mesh) was gently packed dry into a column. The extract to be separated was adsorbed onto some amount of the silica gel and packed on top of it and a cotton wool placed on the packed column. The solvent or mixture of solvents to be used in eluting the chromatographic column was placed on top of the packed column to separate the extract into different fractions and the eluates collected into glass beakers. This method was used in the fractionation of the various extracts and isolation of the compounds.

Thin layer chromatography

The one way ascending technique was used. Samples to be analysed by TLC were dissolved in suitable organic solvent(s) and applied on pre-coated silica gel TLC plates as spots with the aid of capillary tubes at one end of the plate in a straight line, about 2 cm above the bottom edge and 1.5 cm away from the sides. The spots were dried and the plates placed in a chromatank containing the mobile phase that has been prepared in the tank at least 30 minutes earlier. The mobile phase ran along the TLC plate in an ascending manner due to capillary action, carrying with it the components of the extract or the mixture. When the mobile phase reached the desired distance, the plate was removed, the solvent front marked and the plate dried. The separated compounds were identified by observing the chromatogram under ultra-violet light for fluorescence. This was followed by spraying with anisaldehyde in conc. H2SO4 and heating at 120 C. This method was used for all TLC analysis [18].

Chromatographic Fractionation of aqueous extract of *Cestrum nocturnum*

100 g of the water extract was reconstituted in a minimum amount of solvent and adsorbed onto silica gel. A column tube 1ft X 2.5 cm was taken and dried. This column was packed with slurry of silica

gel (70-230 mesh). The lower end of the column was plugged with absorbent cotton. The column was clamped and fitted in vertical position on a stand. The column was then half filled with n-hexane & silica gel slurry poured in small portions and allowed to settle gently till a necessary length of the column was obtained. The extract which was adsorbed in silica gel, then poured onto the bed of silica, covered it again with a layer of cotton wool and more amount of solvent was poured over it. The column was then run by gradient elution technique. aqueous extract was subjected to column chromatography using different solvent systems 100% Hexane, 80% Hexane : 20% Chloroform, 60% Hexane : 40% Chloroform, 40% Hexane : 60% Chloroform, 20% Hexane : 80% Chloroform, 100% Chloroform, 80% Chloroform : 20% Ethanol, 60% Hexane : 40% Ethanol, 40% Hexane : 60% Ethanol, 20% Hexane : 80% Ethanol, 100% Ethanol, 80% Ethanol : 20% Methanol, 60% Ethanol : 40% Methanol, 40% Ethanol : 60% Methanol, 20% Ethanol : 80% Methanol, 100% Methanol, 80% Methanol : 20% Water, 60% Methanol : 40% Water, 40% Methanol : 60% Water, 20% Methanol : 80% Water, 100% Water.

Approximately 80 fractions of 80 ml aliquots were collected. TLC analysis of the eluates led to the bulking of the eluates into 10 fractions. These were labeled AE1 (1.2 g), AE2 (1.8 g), AE3 (5 g), AE4 (4.5 g), AE5 (13.2 g), AE6 (14.3 g), AE7 (2.3 g), AE8 (13.5 g), AE9 (20.1 g) and AE10 (19.2 g). The bulked fractions were screened for activity. Fractions AE6 and AE8 were the most active with AE5 having the highest activity.

Isolation of Compounds from Fraction AE6

Fraction AE6 (14.3 g) was column-chromatographed over silica gel (70-230 mesh). 100% Hexane, 80% Hexane : 20% Chloroform, 60% Hexane : 40% Chloroform, 40% Hexane : 60% Chloroform, 20% Hexane : 80% Chloroform, 100% Chloroform, 80% Chloroform : 20% Ethanol, 60% Chloroform : 40% Ethanol, 40% Chloroform : 60% Ethanol, 20% Chloroform : 80% Ethanol, 100% Ethanol, 80% Ethanol : 20% Methanol, 60% Ethanol : 40% Methanol, 40% Ethanol : 60% Methanol, 20% Ethanol : 80% Methanol, 100% Methanol, 80% Methanol : 20% Water, 60% Methanol : 40% Water, 40% Methanol : 60% Water, 20% Methanol : 80% Water, 100% Water. 50 fractions of 50 ml aliquots were collected. Based on the TLC profiles of the eluates, they were bulked into five fractions labelled 6a (3.9 g), 6b (3.9 g), 6c (2.1 g), 6d (1.7 g) and 6e (1.2 g). 6a was further column chromatographed on silica gel and eluted with 100% Hexane, 80% Hexane : 20% Chloroform, 60% Hexane : 40% Chloroform, 40% Hexane : 60% Chloroform, 20% Hexane : 80% Chloroform, 100% Chloroform, 80% Chloroform : 20% Ethanol, 60% Chloroform : 40% Ethanol, 40% Chloroform : 60% Ethanol, 20% Chloroform : 80% Ethanol, 100% Ethanol, 80% Ethanol : 20% Methanol, 60% Ethanol : 40% Methanol, 40% Ethanol : 60% Methanol, 20% Ethanol : 80% Methanol, 100% Methanol, 80% Methanol : 20% Water, 60% Methanol : 40% Water, 40% Methanol : 60% Water, 20% Methanol : 80% Water, 100% Water. 80 fractions of 10 ml aliquots were collected. Five sub-fractions were obtained after TLC analysis namely; A1, A2, A3, A4 and A5. A2 which was ran with 60 % of Ethanol in the water afforded the compound AE6a (1.2 g) which appeared as single spot with an *Rf* value of 0.46 on TLC analysis using Chloroform : Ethanol : Water (1:6:3) and anisaldehyde as a detecting reagent.

Isolation of compounds from AE5

Fraction AE5 (13.2 g) was column chromatographed on silica gel and eluted with 100% Hexane, 80% Hexane : 20% Chloroform, 60% Hexane : 40% Chloroform, 40% Hexane : 60% Chloroform, 20% Hexane : 80% Chloroform, 100% Chloroform, 80% Chloroform : 20% Ethanol, 60% Chloroform : 40% Ethanol, 40% Chloroform : 60% Ethanol, 20% Chloroform : 80% Ethanol, 100% Ethanol, 80% Ethanol : 20% Methanol, 60% Ethanol : 40% Methanol, 40% Ethanol : 60% Methanol, 20% Ethanol : 80% Methanol, 100% Methanol, 80% Methanol : 20% Water, 60% Methanol : 40% Water, 40% Methanol : 60% Water, 20% Methanol : 80% Water, 100% Water to yield thirty fractions of approximately 50ml aliquots. Following TLC analysis, the fractions were bulked into 6 sub-fractions that were labeled 5a (1.5 g), 5b (1.0 g), 5c (2.4 g), 5d (3.5 g), 5e (1.8 g) and 5f (0.8 g). 5f which showed one compound was washed on a short silica gel column with water to give a AE5a (10 mg). The *Rf* value was found to be 0.48 after analysis was done on TLC plate and ran with Chloroform : Ethanol : Water (1:4:5).

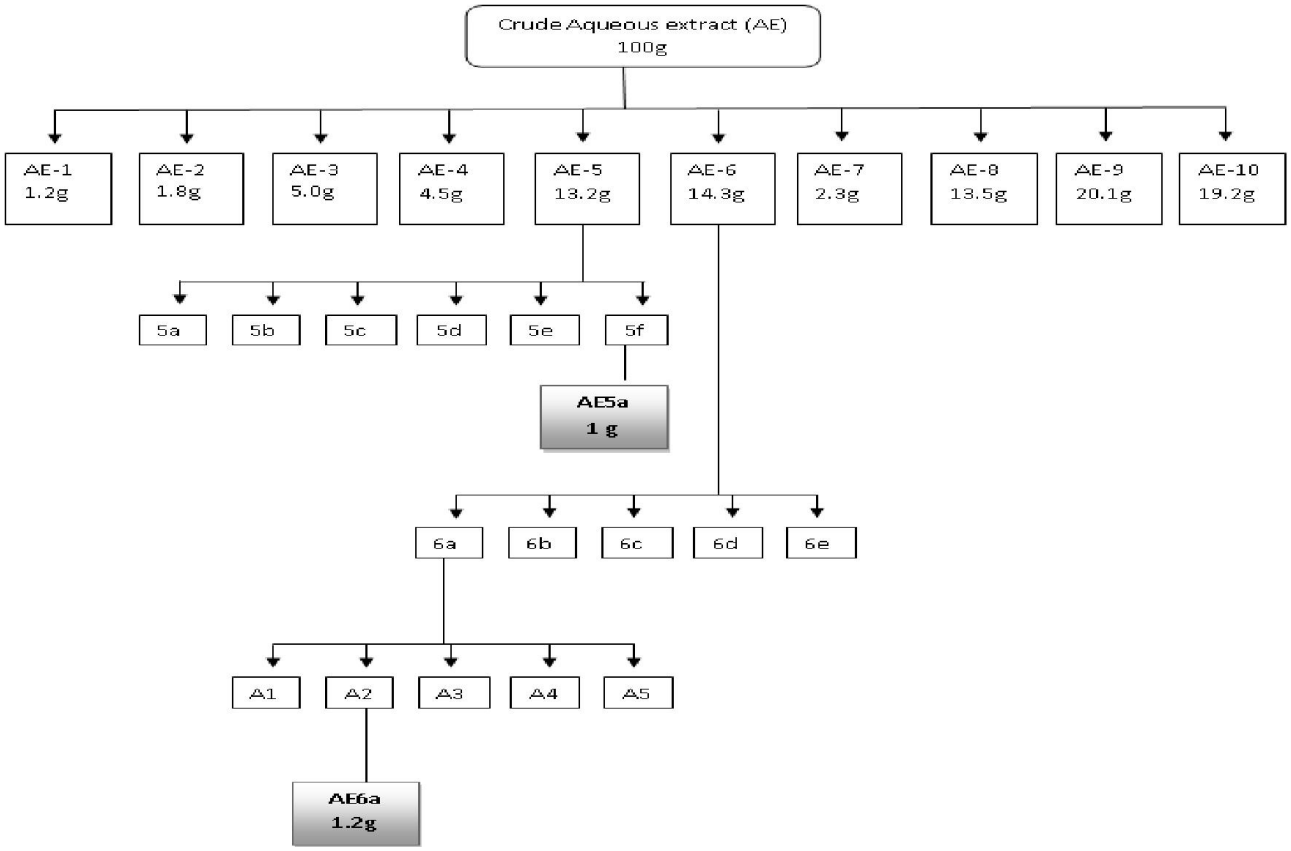

Figure 1. A schematic representation of the fractionation and isolation of compounds from the aqueous extract of the *Cestrum nocturnum*

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) [19].

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 µL) was mixed with 10 µL of samples (0.1 – 2 µg/µL) and incubated at 37 C for 5 min. Then, 200 µL 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 (aPPT) 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 µL) was mixed with 10 µL of samples (0.1 – 2 µg/µL) 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 µl 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 µl) 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.

Characterization of the Isolated Compounds By IR Spectroscopy

The presence of functional group in the isolated compounds was determined by IR spectroscopy. Two mg of sample was taken and mixed with one mg of potassium bromide. Then it was kept under IR light.

Characterization of the isolated compounds by N.M.R. Spectroscopy

The isolated compounds were subjected to ^{1}H-NMR and C-NMR analysis by using $CDCL_3$, DMSO, and D_6 as a solvent.

Characterization of The Isolated Compounds By Mass Spectroscopy

LC-MS of the isolated compound was done with the help of Electro spray ionization (ESI) Bombardment Technique.

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. Percentage Platelet aggregation & inhibition results of Isolated fractions (AE1 to AE10) of aqueous extract of *Cestrum nocturnum* (n=5)

Treatment	Dose	% inhibition
Vehicle control (D/W)	--	--
Heparin	(20 µg/ml)	50.4 ± 3.2
AE1	50 µM	30.8±1.92
AE1	100µM	35±2.91
AE2	50 µM	36±3.16
AE2	100µM	38.4±2.88
AE3	50 µM	38.8±1.78
AE3	100µM	41±2.64
AE4	50 µM	37.6±3.20
AE4	100µM	40.8±3.27
AE5	50 µM	43.8±3.70
AE5	100µM	49.2±1.30*
AE6	50 µM	42.2±2.58
AE6	100µM	46.8±3.63*
AE7	50 µM	39.8±2.58
AE7	100µM	41.4±3.91
AE8	50 µM	42.2±3.63
AE8	100µM	42.8±3.70
AE9	50 µM	32.8±3.42
AE9	100µM	44±4.18
AE10	50 µM	37.8±1.30
AE10	100µM	41.6±1.14

Values are expressed as mean ± SD. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to standard (n=5)

The effect on the *in vitro* platelet deaggregation effect of isolated fraction of (AE1 to AE10) aqueous extract of *Cestrum nocturnum* is presented in Table 2. The platelet deaggregation activity of the isolated fraction were evaluated by using two concentration i. e. 50 µM &100 µM. from the result which were obtained it can be say that the results were migrating around the previous result obtained with the concentration of 10mg/mL of the aqueous extract of the plant and the result were not following any type of pattern & we can say that the result were in random fashion. But on further statistical analysis it was found that the among all the isolated fractions only the AE5 and AE6 were much effective as compared to the other isolated fractions and shows the significant result and the

concentration of 100 µM when compared to the standard (streptokinase). But on with close observation it observed that the when we are moving from AE1 to AE10 the percentage of platelet deaggregation were increasing but the sequence had disturbed at AE5 and AE6. And when comparing all the isolated fraction at 50 µM & 100µM, it was observed that the always 50µM concentration shows less percentage of platelet deaggregation as compared to the 100µM percentage of platelet deaggregation. And overall the AE5 at 100µM and AE6 100µM shows the significant result as depicted in the table no. 2.

Table 3. Effect of different isolates fractions of aqueous extract and the standard on *in vitro* clot lysis (AE1 to AE10) of *Cestrum nocturnum*

Treatment	Dose	% Clot lysis (mean ± S.D)
Streptokinase (standard)	100µl	48.4 ± 1.14
AE1	50 µM	11.2±2.58
AE1	100µM	16.4±2.07
AE2	50 µM	11.2±2.48
AE2	100µM	16.2±2.77
AE3	50 µM	11.4±2.19
AE3	100µM	23±3.39
AE4	50 µM	11.4±2.50
AE4	100µM	20.2±1.78
AE5	50 µM	22.8±1.92
AE5	100µM	43±5.95*
AE6	50 µM	11.2±2.58
AE6	100µM	41±3.67*
AE7	50 µM	12.2±1.92
AE7	100µM	23.8±3.11
AE8	50 µM	12.4±2.07
AE8	100µM	22.2±3.03
AE9	50 µM	13.4±3.71
AE9	100µM	24.2±3.11
AE10	50 µM	14.6±2.70
AE10	100µM	22.4±2.07

Statistical representation of percent clot lysis by different treatments, and negative control (sterile distilled water) done by paired t-test analysis; clot lysis % is represented as mean ± S.D. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to standard (n=5)

The effect on the *in vitro* clot lysis effect of isolated fraction of (AE1 to AE10) aqueous extract of *Cestrum nocturnum* is presented in Table 3. The clot lysis activity of the isolated fraction were evaluated by using two concentration i. e. 50 µM &100 µM. from the result which were obtained it can be say that the results were migrating around the previous result obtained with the concentration of 10mg/mL of the aqueous extract of the plant and the result were not following any type of pattern & we can say that the result were in random fashion. But on further statistical analysis it was found that the among all the isolated fractions only the AE5 and AE6 were much effective as compared to the other isolated fractions and shows the significant result and the concentration of 100 µM. But on with close observation it observed that the when we are moving from AE1 to AE10 the percentage of clot lysis were increasing but the sequence had disturbed at AE5 and AE6. And when comparing all the isolated fraction at 50 µM & 100µM, it was observed that the always 50µM concentration shows less percentage of clot lysis as compared to the 100µM percentage of clot lysis. And overall the AE5 at 100µM and AE6 100µM shows the significant result as depicted in the table no. 3.

Table 4. Anticoagulant activity of isolated fractions of aqueous extract of *Cestrum nocturnum*

Sample	Dose	aPTT(Seconds)	PT (Seconds)	TT (Seconds)
Control	Saline	37 ± 1.58	15.2 ± 1.92	8±0.7
Heparin	1 IU/mL	113.2 ± 1.64	39.4 ± 2.40	20.4 ± 2.07
AE1	50 µM	69 ± 6.0	23.2±2.28	15±1.58
AE1	100µM	75.6±2.30	23.8±3.03	13.8±1.92
AE2	50 µM	70 ±3.16	22.4±2.70	13±1.58
AE2	100µM	74.2±4.43	26.4±2.07	14±1.58
AE3	50 µM	66.4±2.96	25.4±2.88	12.6±2.30
AE3	100µM	74±2.91	32.6±2.07	13.6±2.07
AE4	50 µM	79.6±2.30	25.2±1.48	12.4±2.40
AE4	100µM	90.4±1.67	32.8±2.48	11.8±2.58
AE5	50 µM	82.2±1.48	29±2.73	13.4±1.94
AE5	100µM	98.2±2.68*	36.2±2.58*	16.8±1.30*
AE6	50 µM	73±4.52	26.8±1.483	11.8±1.92
AE6	100µM	87.4±2.70*	36±2.23*	14.4±1.51*
AE7	50 µM	69.8±2.77	23±1.58	12±1.58
AE7	100µM	79.8±2.77	24.6±1.14	13.6±1.14
AE8	50 µM	76.6±2.30	26.8±1.92	11.2±1.30
AE8	100µM	87±4.84	32.4±2.96	13±1.58
AE9	50 µM	67.4±2.30	24±2.12	11.6±1.14
AE9	100µM	80.2±3.34	26.2±2.68	13±2.54
AE10	50 µM	70.2±3.11	23.8±1.92	11±1.58
AE10	100µM	80.2±3.03	25.2±2.58	11.2±1.92

Values are expressed as mean ± SD. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to control (n=5)

The effect on the *in vitro* study of aPTT, PT and TT seconds of isolated fraction of (AE1 to AE10) aqueous extract of *Cestrum nocturnum* is presented in Table 4.The isolated fraction were evaluated by using two concentration i. e. 50 µM &100 µM. from the result which were obtained it can be say that the results were migrating around the previous result obtained with the concentration of 10mg/mL of the aqueous extract of the plant and

the result were not following any type of pattern & we can say that the result were in random fashion. But on further statistical analysis it was found that the among all the isolated fractions only the AE5 and AE6 were much effective as compared to the other isolated fractions and shows the significant result with the concentration of 100 µM. But on with close observation it observed that the when we are moving from AE1 to AE10 the percentage of clot lysis were increasing but the sequence had disturbed at AE5 and AE6. And when comparing all the isolated fraction at 50 µM & 100µM, it was observed that the always 50µM concentration shows less time of aPTT, PT and TT as compared to the 100µM. And overall the AE5 at 100µM and AE6 100µM shows the significant result as depicted in the table no. 4.

Table 5. Percentage Platelet aggregation & inhibition results of Isolated fraction AE5a of aqueous extract of *Cestrum nocturnum* (n=5)

Treatment	Dose	% inhibition
Vehicle control (D/W)	--	--
Heparin	(20 µg/ml)	52.2 ± 2.387
AE5a	100µM	38.6±2.88
AE5a	200 µM	44.2±2.588
AE5a	300 µM	51±1.58*

Values are expressed as mean ± SD. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to standard (n=5)

Table 6. Effect of different isolate fractions (AE5a) of aqueous extract and the standard on *in vitro* clot lysis

Treatment	Dose	% Clot lysis (mean ± S.D)
Streptokinase (standard)	100µl	47.6 ± 1.14
AE5a	100 µM	13±1.58
AE5a	200µM	23.4±2.07
AE5a	300 µM	44±2.34*

Statistical representation of percent clot lysis by different treatments, and negative control (sterile distilled water) done by paired t-test analysis; clot lysis % is represented as mean ± S.D. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to standard (n=5)

Table 7. Anticoagulant activity of isolated fraction (AE5a) of aqueous extract of *Cestrum nocturnum*

Sample	Dose	aPTT (Seconds)	PT (Seconds)	TT (Seconds)
Control	Saline	36.2 ± 1.30	14.8 ± 0.83	6.6±1.14
Heparin	1 IU/mL	114.2 ± 1.30	44.6 ± 1.14	18 ± 0.70
AE5a	100 µM	65 ±1.58	24.8±2.38	13.2±1.30
AE5a	200µM	82.2±1.92	29.2±2.38	14.4±1.14
AE5a	300 µM	95.8±1.92*	39.8±2.86*	14.4±1.51*

Values are expressed as mean ± SD. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to control (n=5)

Table 8. Percentage Platelet aggregation & inhibition results of Isolated fractions (AE6a) of *Cestrum nocturnum* (n=5)

Treatment	Dose	% inhibition
Vehicle control (D/W)	--	--
Heparin (20 µg/ml)	(20 µg/ml)	50.4 ± 1.14
AE6a	100µM	33±1.58
AE6a	200 µM	38.8±1.64
AE6a	300 µM	47.2±1.48*

Values are expressed as mean ± SD. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to standard (n=5)

Table 9. Effect of different isolate fractions (AE6a) of aqueous extract and the standard on *in vitro* clot lysis of *Cestrum nocturnum*

Treatment	Dose	% Clot lysis (mean ± S.D)
Streptokinase (standard)	100µl	49.2 ± 0.83
AE6a	100 µM	21.2±1.92
AE6a	200µM	33.4±2.07
AE6a	300 µM	46.8±1.48*

Statistical representation of percent clot lysis by different treatments, and negative control (sterile distilled water) done by paired t-test analysis; clot lysis % is represented as mean ± S.D. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to standard (n=5)

Table 10. Anticoagulant activity of isolated fraction (AE6a) of aqueous extract of *Cestrum nocturnum*

Sample	Dose	aPTT (Seconds)	PT (Seconds)	TT (Seconds)
Control	Saline	36.2 ± 1.30	14.4 ± 1.14	6.6±1.14
Heparin	1 IU/mL	114.2 ± 1.30	43.4 ± 1.51	18.8 ± 0.83
AE6a	100 µM	68 ± 1.58	26.6±1.81	13.2±1.30
AE6a	200µM	82.6±2.40	29±3.16	13.8±0.83
AE6a	300 µM	87.8±1.64*	28±3.16*	15±1.58*

Values are expressed as mean ± SD. p 0.05(*) or p 0.01(**) or p 0.001(***) compared to control (n=5)

Table no 5,6 and 7 shows the patelet deaggregation, clot lysis and anticoagulant activity (aPTT, PT and TT) results of isolated fraction AE5a in three different concentration i.e. 100µM, 200 µM and 300 µM, on applying the statistical analysis the 300 µM concentration shows the significant result and also the results showing the concentration dependent result.

Table no 8,9 and 10 shows the patelet deaggregation, clot lysis and anticoagulant activity (aPTT, PT and TT) results of isolated fraction AE6a in three different concentration i.e. 100µM, 200 µM and 300 µM, on applying the statistical analysis the 300 µM concentration shows the significant result and also the results showing the concentration dependent result.

Identification and characterization

Four isolated compounds were identified spectral data of IR, Mass, and 1H and 13C NMR.

Rutin

The IR spectrum of the AE5a is shown in Fig. 2. IR values: 3483 OH (bonded), 2931 CH stretch, 1669 C=O, 1504 C=C aromatic, 1348 C-O-C, 1204 C-O-C, 1043 C-O-C. The mass spectrum of AE5a is illustrated in Figure.3. The AE5a compound gave a fragments peaks at [M+H]+=611, [M+H]+- Rhamnose=465, [M+H]+-Rhamnose-Glucose=303. This corresponds to C27H30O16. The NMR data of the compound depicting the structure of rutin were as follows 1H-NMR (Figure. 4) (500 MHz, DMSO-d6), ppm: 3.40-3.72 (m, 12H of sugar moieties), 3.86 (d, 1H-Rham), 1.03 (3H, d, CH3-Rham), 4.06-4.83 (4H, H-1 Glu), 5.32 (1H, d, H-6), 6.18 (1H, d, H-8), 6.42 (1H, d, H-5'), 7.85 (1H, m, H-2', H-6'); the 13C NMR (Figure.5) spectrum (500 MHz, DMSO-d6) showed 29 carbon signals which indicated the presence of 15 carbon signals due to the flavonol skeleton. The spectrum of the isolated compound, revealed the presence of one methyl carbon (C=18.26 ppm) of rhamnose, one methylene carbon (C=67.36 ppm), 15 methene carbons and 10 quaternary carbons. In the aliphatic region of 13C NMR, 12 carbon resonances are assigned for a rutinoside moiety among which the most downfield signals at 102.16 and 101.38 are assigned for the two anomeric carbons C1''' and C1'' of rhamnose and glucose, respectively . So the isolated compound AE5a was confirmed as rutin.

Quercetin

The IR spectrum of the AE6a is shown in Figure. 6. The absorption around 3350/cm is due to the presence of phenolic hydroxyl groups in the compound. The intense absorption band at 1673/cm is due to the presence of (C=O). The band around 1489/cm is due to the occurrence of the aromatic group in an isolated compound. The mass spectrum of AE6a is illustrated in Figure. 7. The AE6a compound gave a fragments peaks at [M+H-H2O]+=289. [M+H-H2O-CO]+=257. [M+HH2O-2CO]+=229. [M+H-CO]+=275. [M+H-2CO]+=247. This corresponds to $C_{15}H_{10}O_7$. The NMR data of the compound depicting the structure of quercetin. 1H NMR spectra (500 MHz, DMSO-d6) : 6.80 (2H, d, H-6), 6.54 (1H, d, H-8), 7.07 (1H, d, H-2'), 6.79 (1H, d H-5'), 6.85 (1H, d, H-6') (Fig. 8). 13C NMR (500 MHz, DMSO-d6) : 97.65 (CH, C-8), 102.15 (CH, C-6), 104.7 (C, C-10), 116.63 (CH, C-2', C-5'), 119.81 (CH, C-6'), 126.05 (C, C-1'), 132.93 (C, C-3), 146.45 (C, C-3'), 159.71 (C, C-9), 164.20 (C, C-5), 166.74 (C, C-7), 192.63 (C, C-4) (Figure. 9).

Conclusion

From all the result it can be concluded that water extract of *Cestrum nocturnum* plant was found to be best among all the extract under study and from the isolates of water extract of *Cestrum nocturnum* plant AE5 was found to be best activity (anti-platelet, Fibrinolytic and anti-coagulant). Further fractionation of water extract generated two isolates AE5a & AE6a and after their pharmacological activity they wee found to be potent phytochemical constituents. From the structural elucidation study of AE5a & AE6a were identified as Rutin and quercetin respectively.

From this study it was concluded that the flavoinoidal compound i.e. Rutin and Quercetin were present in the *Cestrum nocturnum* plant and they both responsible for the anti-platelet, Fibrinolytic and anticoagulant activity of plant.

Conflicts of interests

The authors declare no conflicts of interests.

References

[1]. Baquar SR. The role of traditional medicine in rural environment. In: Issaq S (ed) Traditional Medicine in Africa. East Africa Educational Publishers Ltd., Nairobi 1995; 141-142.

[2]. Abalson PH. Medicine from plants. Science, 1990; 247: 513-519.

[3]. Lanfranco G. Invited review article on traditional medicine. Electronic Journal of Biotechnology1999; 2: 1-3.

[4]. World Health Organization. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. WHO, Geneva, Switzerland 2001.

[5]. Edoega HO, Mbaeble BO and Okwu DE. Phytochemical constituents of some Nigerian medicinal plants, African Journal of Biotechnology. 2005; 4:685-688.

[6]. Rahman KH and Billington D. Dietery supplementation with aged garlic extract inhibits ADP-induced platelet aggregation in humans. Human nutrition and metabolism research communication. 2000;130: 2662-2665.

[7]. Dutta-Roy AK, Crosbie L and Gordon MJ. Effect of tomato extract on human platelet aggregation *in vitro*, Platelet. 2001;12: 218-227.

[8]. Hutchings A. Zulu medicinal plants: an inventory, 1st ed, PMB University of Natal Press, 1992.

[9]. Ichinose, A. Multiple members of the plasminogen-apolipoprotein (a) gene family associated with thrombosis. *Biochemistry*, 1996; 31, 3113–3118.

[10]. Sofoware A. Medicinal plants and traditional medicine. Spectrum books Ltd. Ibadhan, Nigeria: 1993;289.

[11]. Amrani S. Harnafi H. Gadi D. Mekhfi H. Legssyer A. Aziz M. Martin-Nizard F. Bosca L. Vasorelaxant and anti-platelet aggregation effects of aqueous *Ocimum basilicum* extracts. Journal of Ethnopharmacology 2009;125(1): 157-162.

[12]. Gadi D and Bnouham M. Parsley extracts inhibits *in vivo* and *ex vivo* platelet aggregation and prolongs bleeding time in rats, Journal of ethnopharmacology. 2009;125:170-174.

[13]. Lau AJ, Toh DF, Chua TK, Pang YK, Woo SO and Koh HL. Antiplatelet and antiocoagulant effects of *Panaxnoto ginseng*. . Journal of Pharmacology. 2009;125: 380-38.

[14]. Goldhaber SZ and Morrison RM. Pulmonary embolism and deep vein thrombosis. Circulation. 2002: 106: 1436–1438.

[15]. Van Wyk B-E, Van Oudtshoom and B, Gerike N. Medicinal plants of South Africa, Briza, *Pretoria.* 1997; 8(12) 14-20.

[16]. Cooper EL. Drug discovery, CAM and natural products. eCAM 2001;1: 215-217.

[17]. Lindequist U, Niedermeyer T, Jülich WD. The pharmacological potential of mushrooms. eCAM, 2005; 2: 285-299.

[18]. Kokate CK. A text book of Practical pharmacognosy 5th Edition, Vallabh Prakashan New Delhi: 2005;107-111.

[19]. Rashid HU, Rahman MA, Sultana R, Bin Emran T, Islam MS, Chakma JS, Hasan CM. Effects of organic extracts of six Bangladeshi plants on *in vitro* thrombolysis and cytotoxicity. BMC Complement Altern Med. 2013; 13, 25.

[20]. Mao W, Li H, Li Y, Zhang H, Qi X, Sun H, Chen Y, Guo S. Chemical characteristic and anticoagulant activity of the sulfated polysaccharide isolated from *Monostroma latissimum* (Chlorophyta). Int J BiolMacromol, 2009;44:70–74.

[21]. Sahane RS, Wankhade P. A, Shrungarpure MA. Pharmacological and phytochemical investigation of *Cestrum nocturnum* leaf extract for antihyperglycemic and antihyperlipidemic activity. International Journal of Research in Pharmacology & Pharmacotherapeutics, 2014;4, 255-268.