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PRELIMINARY PHYTOCHEMICAL SCREENING AND *IN VITRO* ACTIVITIES OF BOUGAINVILLEA CASCADE FLOWER EXTRACT

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Anti diabetic activity, Glucose diffusion method, *Bougainvillea cascade,* Heamoglobin glycosylate, Anti inflammatory activity, Thrombolytic activity.

ABSTRACT

The leaves of *Bougainvillea cascade* extract possesses several bioactivities such as antiulcer, anti diarrheal, anti bacterial, anti diabetic and anti hepatic and is used in traditional medicinal systems. Its flowers are used for lowering blood pressure. The present work was carried out to evaluate antidiabetic, anti-inflammatory and thrombolytic activity of petroleum ether, ethylacetate, chloroform, methanolic and aqueous extracts of *Bougainvillea cascade* (family: Nyctagainacea). Initially we perform preliminary phytochemical screening for all extracts and get various chemical constituents of therapeutically importance. For invitro antidiabetic activity petroleum ether and ethyl acetate extract showed potential inhibition of glucose diffusion using supplements useful for type-2-diabetes. Another invitro anti diabetic activity by haemoglobin glycosylation showed petroleum ether extract, ethyl acetate extract of the plant exhibited higher inhibition of glycosylation as compared with standard Metformin. For invitro anti-inflammatory activity chloroform and aqueous extracts showed significant activity when compared with the standard Diclofenac sodium. In addition invitro thrombolytic activity of aqueous and chloroform extracts of the same plant showed significant activity against streptokinase.

INTRODUCTION:

Bougainvillea cascade(family: Nyctagainacea) is an ornamental shrubby climbing plant that is widely cultivated in the tropics, locally known as "Paper flower", is a great white variety with pure white bracts and the plant has a beautiful cascading habit with immense medicinal properties[1,2]. It is indigenous to tropical countries and is considered as an important folklore medicine. In the traditional system of medicine, there is a greater demand for leaf extract of Bougainvillea species in treatment for different kinds of illness such as anti-ulcer[3], anti-diarrhoeal, antibacterial[4], anti-hepatitic, antiviral5, antidiabetic activity[6]and anti-inflammatory[7]. Its flowers are used as treatment for low blood pressure. Not much work has been carried out on in vitro activities of the flower of *Bougainvillea cascade* and hence we tried to identify the phytoconstituents responsible for the biological activities of different solvent extracts of *Bougainvillea cascade.*

MATERIALS AND METHODS:

Plant material:

The *Bougainvillea cascade* flowers were collected in 22-1-2016 at our college garden Tadepalligudem west Godavari district, India. The plant material was identified and authenticated by Department of Botany, K.G.R.L College, Bhimavaram.

Preparation of extracts:

The shade dried powdered form of *Bougainvillea cascade* flowers were taken and subjected to continuous percolation process with petroleum ether and ethyl acetate, chloroform and methanol separately in a soxhlet apparatus and the remaining residue was macerated with water. Each extracts were concentrated by distilling off the solvent and then evaporated to dryness. The yield of extracts was found to be 50g/l,54.3g/l, 50g/l, 50g/l and 20.6g/l respectively The obtained extracted fractions were dissolved in 1% carboxy methyl cellulose (CMC) and were used for the present study.

PHYTOCHEMICAL ANALYSIS:

The phytochemical analysis of methanolic, chloroform, aqueous extracts of flowers of *Bougainvillea cascade L* was analysed for the compounds such as tannins, saponins, flavonoids, phenols, terpenoids, glycosides and alkaloids. (Table.1, fig.1). In the present study the phytochemicals occurring in the various solvent extracts of *Bougainvillea cascade* flowers (Methanolic, chloroform and aqueous extracts) were analyzed qualitatively by phytochemical screening. The results revealed the presence of various secondary metabolites of therapeutical importance. The major phytochemicals found were tannins, saponins, flavonoids, terpenoids, cardiac glycosides and alkaloids.

EXPERIMENTAL:

In Vitro Anti diabetic activity

A) Effects of Various Extracts on In vitro Inhibitory Glucose Diffusion[8]:

This method is simple to evaluate the effect of *Bougainvillea cascade* flowers extract on glucose diffusion in vitro. The model was adapted from a method described by Edwards[9]et al., which involved the use of a sealed dialysis tube into which 15ml of a solution of glucose and sodium chloride (0.15M) was introduced and the other appearance of glucose in the external solution was measured. The model used in the present experiment consisted of a dialysis tube (6cm x15mm) into which 1ml of 50g/l of all the five plant extracts dissolved in 1% CMC and 1ml of 0.15M sodium chloride containing 0.22M D-glucose were added. The dialysis tube was sealed at both ends and was placed in a 50ml centrifuge tube containing 45ml of 0.15M sodium chloride and then the tubes were placed on an orbital shaker and kept at room temperature. The diffusion of glucose into the external solutions was monitored at set time intervals.

Effect on Glucose Diffusion:

Nature was gifted with discrete flora with distinctive medical opinions. A natural medicine originated from herbs offers good clinical opportunities and shows a bright future in the therapy of diabetes mellitus and its complications. The effect of *Bougainvillea cascade* flowers as anti- diabetic agents has been studied in which all extracts showed varying effect on glucose utilization. But, the pet-ether, ethyl acetate extracts produced a significant decrease in glucose concentration during the experiment. The effects of *Bougainvillea cascade* flower extracts on glucose diffusion inhibition were summarized in table 2. At the end of 27 hrs, glucose movement of control

(without plant extract) in the external solution had reached a plateau with a mean glucose concentration above 300mg/dl (320.2±0.43). It was evident from the table 2 that the pet-ether, ethyl acetate extracts were found to be potent inhibitors of glucose diffusion ($p<0.001$) compared to control. The pet-ether, ethyl acetate extracts were found to be more potent than other extracts showing the lowest mean glucose concentration of 151.40+0.8mg/dl,214±1.32 mg/dl at the end of 27 hrs (Table.2)

Results and Discussion:

Diabetes mellitus is a life threatening disorder with increasing incidence throughout the world. In 2014 the global prevalence of diabetes was estimated to be 9% among adults aged 18+ years. WHO projects that diabetes will be the 7th leading cause of death in 2030[10]. Anti hyperglycemic activities of most effective plants were in part explained by the ability of the phytoconstituents to increase glucose transport and metabolism in muscle and or to stimulate insulin secretion[9]. In the present study, research has been carried out to evaluate the potential of various extracts of *Bouganvillea cascade* to additionally suppress the diffusion and movement of glucose in the intestinal tract [11, 12, 13]. A decoction of *Bougainvillea* flower extract is used worldwide for the treatment of various ailments including antidiabetic.. Numerous alkaloids, flavanoids, sterols, polyphenolic compounds, triterpenoids and other chemical compounds in the plant may account for the observed antidiabetic effects of the flower extracts.

Statistical Analysis: Data was expressed as mean + S.E.M. Statistical comparisons between groups were done by one way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparison tests to analyze the differences. $p<0.001$ were considered as significant.

The present study reveals that the ability of different extracts of *Bougainvillea cascade* flower to inhibit glucose diffusion using an in vitro model of glucose absorption. In particular, pet-ether, ethyl acetate extracts show potential inhibitory of glucose diffusion supplements useful for type 2 diabetes. Further studies are required to elucidate whether in vitro effects represent therapeutic potential by limiting postprandial glucose absorptions and for improving glycemic control in type 2 diabetic subjects.

B) Evaluation of haemoglobin glycosylation:

Preparation of haemoglobin glycosylate: The blood was collected from a healthy human volunteer and transferred into a blood bottle containing an anticoagulant. Hemolysate was prepared based on the principle of hypotonic lysis[10]. The red blood collected were washed thrice with 0.14M NaCl solution and one volume of red blood cells suspension was lysed with two volumes of 0.01M phosphate buffer, pH 7.4 and 0.5 volume of CCl_4. The haemolysate was then freed from the debris by centrifugation at 2300 rpm for 15 min at room temperature. The haemoglobin rich fraction ie the upper layer was separated and dispensed into sample bottle for storage and refrigerated until required for use[14].

Estimation of haemoglobin glycosylation :1 mL the above prepared each of haemoglobin glycosylate was transferred into three test tubes, each containing 1 mL solution of different concentrations (2, 10, and 20 mg/mL) of glucose in 0.01M phosphate buffer (pH 7.4). The contents were incubated at room temperature for 72 hrs. A blank solution in which the addition of glucose solution was omitted and was used as the control. The amounts of hydroxymethylfurfural in nanomole released were estimated at different incubation periods of 0, 24 hrs, 48 hrs and

72 hrs which correspond to the degree of glycosylation[15].

Effect of Extracts on Haemoglobin Glycosylation:

To 1 mL of haemoglobin glycosylate solution, 5µL of Gentamycin and 25 µL of the petroleum ether and ethylacetate plant extracts (30µg/mL) were added. The reaction was started by the addition of 1 mL of 2% glucose in 0.01M phosphate buffer (pH 7.4) and incubated in the dark at room temperature. The concentrations of glycated haemoglobin at the incubation period of 0, 24, 48 and 72 hrs were estimated spectophotometrically at 520nm[15].

Effect of extract at physiological glucose concentration:

Take nine test tubes separately to first three test tubes (1, 2, 3) add 1 mL of haemoglobin glycosylate solution, 1 mL of glucose solution (2mg, 10mg and 20mg in 20 mL each of 0.01M phosphate buffer, pH 7.4) and 5µL of gentamycin dissolved in 0.01M phosphate buffer (pH 7.4) were mixed and incubated in the dark at room temperature for 72 hours in the presence 30 µg/mL of Metformin. In next three test tubes (4, 5, 6) containing 1 mL of haemoglobin glycosylate add 2mg, 10mg and 20mg of ethylacetate extracts dissolved in 1%CMC seperately,to them add 20ml of 0.01M phosphate buffer separately in each test tube and 5µL of gentamycin dissolved in 0.01M phosphate buffer (pH 7.4) each in all the above test tubes (Ph 7.4) and incubated for 72hrs. In remaining three test tubes (7, 8, 9) containing 1 mL of haemoglobin glycosylate add 2mg, 10mg and 20mg of petroleum ether extracts dissolved in 1%CMC separately, to them add 20ml of 0.01M phosphate buffer separately .in each test tube and 5µL of Gentamycin dissolved in 0.01M phosphate buffer (pH 7.4) each in all the above test tubes (Ph 7.4) and incubated for 72hrs.

All the above nine Haemoglobin glycosylate concentrations were estimated spectrophotometrically at 520 nm, as an index for measuring the degree of haemoglobin glycosylation. The assay was carried out in triplicates (Adisa *et al*., 2004). Increased concentration of glucose in the blood leads to its binding to hemoglobin which may result in the formation of the reactive oxygen species. Plant extracts play an important role the inhibition of the glycosylation end products. An increase in the glycosylation was observed on incubation of hemoglobin with the increasing concentration of the glucose (2mg, 10mg and 20mg) over a period of 72hrs. However, the plant extracts significantly inhibited the haemoglobin glycosylation which is indicated by the presence of increasing concentration of haemoglobin (Table3, Fig2). Petroleum ether extract of *Bougainvillea cascade* exhibited higher inhibition of glycosylation as compared with the standard Metformin and ethylacetate extract of same plant. The plant extracts also displayed the inhibition of haemoglobin glycosylation at different physiological concentrations of the glucose over the period of 72 hours indicating that the plant extracts decreases the formation of the glucose- haemoglobin complex and thus amount of free haemoglobin increases. The results indicate that Petroleum ether and ethyl acetate extracts of Bougainvillea cascade has appreciable antidiabetic activity as compared to standard Metformin.

CONCLUSION

The antidiabetic properties of plants can be evaluated in vitro by several methods such as Effect on Glucose Diffusion, effect on glycosylation of the haemoglobin. It was observed that the plant extracts shows significant effect on glucose diffusion and inhibited glycosylation of haemoglobin and thereby helps in the inhibition of the formation of glycated end products. We can therefore

conclude from this study that the presence of the phytochemicals in these plants might be the reason for these inhibitions and that the plants may essentially contain herbal bioactive compounds which require further structural elucidation and characterization methodologies to identify the bioactive constituents. Further ex vivo and in vivo investigations should be done for confirming the anti diabetic activity of these plants. The plant extracts understudy can serve as therapeutic agents and can be used as potential sources of novel bioactive compounds for treating Diabetes mellitus type 2.

IN VITRO ANTI-INFLAMMATORY ACTIVITY:

Blood sample:

Blood samples were collected from healthy human volunteers (n=3) by maintaining aseptic condition without a history of oral contraceptive or NSAIDS. 1ml of blood was collected (fig3) from each volunteer according to protocol accepted by institutional committee of Sri Vasavi institute of pharmaceutical sciences. Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid and 0.42 % sodium chloride in water). Theblood was centrifuged at 3000 rpm for 10 min and packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

Heat Induced Hemolysis:

The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis[16]. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hyposaline [0.36 %], 0.5 ml HRBC suspension [10 % v/v] with 0.5 ml of plant extracts and standard drug diclofenac sodium of various concentrations (100, 200,300,400, 500µg/ml) and control (distilled water instead of hypo saline to produce 100 % hemolysis) were incubated at 37ºC for 30 min and centrifuged respectively. The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100 %. The percentage of HRBC membrane stabilization or protection was calculated by using the formula. The percentage of hemolysis of HRBC membrane can be calculated as follows:

% Hemolysis = (Optical density of Test sample / Optical density of Control) X 100

The percentage of HRBC membrane stabilisation can be calculated as follows:
% Protection = 100 – [(Optical density of Test sample / Optical density of Control) X100]

RESULTS AND DISCUSSION:

The lysosomal enzymes released during inflammation produce a variety of disorders. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation. The non steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane[17].Since HRBC membrane are similar to lysosomal membrane components the prevention of hypotonicity induced HRBC membrane lysis is taken as a measure of anti inflammatory activity of drugs. The results were reported in table 4, 5&fig6. It was observed from the table1 and figure 1 that the chloroform and aqueous extracts show significant anti inflammatory activity at the concentration of 500µg/ml which is comparable to the standard drug Diclofenac sodium. The anti inflammatory activity of the extracts were concentration dependent, with the increasing

concentration the activity is also increased. The chloroform extract of *Bouganvillea cascade* has significant anti inflammatory activity in comparison to the aqueous extract of the same plant. The chloroform extract and aqueous extract shows significant anti inflammatory activity in comparison to the standard Diclofenac sodium.

CONCLUSION:

The extracts exhibited membrane stabilization effect by inhibiting hypo tonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is analogues to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membrane. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bacterial enzymes and proteases which cause further tissue inflammation and damage. From the above study it was concluded that the chloroform extract of *Bouganvillea cascade* has significant membrane stabilization property compared to the aqueous extract of the same plant and it was comparable to the standard drug Diclofenac sodium.

IN VITRO THROMBOLYTIC ACTIVITY:

Preparation of sample: The thrombolytic activities of all aqueous and chloroform extracts were evaluated by a method using streptokinase (SK) as a reference standard. 100gm of chloroform and 100mg of aqueous extract (fig7) of Bougainvillea cascade were dissolved in 10 ml of chloroform and distilled water respectively. They were kept overnight. Then the solution were filtered and further used for study.

Streptokinase (SK): Streptokinase thromboflux (15, 00,000 I.U.,) used as a standard which was collected from Bharat serum and vaccines Ltd, Ambernath. 5 ml sterile distilled water was added to streptokinase vial and mixed properly. From this suspension100µl (30,000 I. U) was used for in vitro thrombolytic activity.

Blood sample: Blood samples were collected from healthy human volunteers (n=3) by maintaining aseptic condition without a history of oral contraceptive or anticoagulant therapy. 1ml of blood was collected from each volunteer according to protocol accepted by institutional committee of Sri Vasavi institute of pharmaceutical sciences.

Thrombolytic activity: Micro centrifuge tubes were taken and empty weight of each tube was noted (fig 8). Note it as W1. Then 0.5ml of blood was transferred to each tube and it is allowed to incubate at 37 °C for 45 minutes for clot formation. After clot formation, fluid was completely released from each microcentrifuge tubes (Fig: 9) and weighs the tubes along with the clot formed[18, 19]. Note it as W2 As a positive control, 100 µl of streptokinase (SK) was used and as a negative non thrombolytic control, 100 µl of distilled water was used. 100 µl of each samples (chloroform and aqueous) were separately added to the micro centrifuge tubes containing clot. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released fluid was discarded and tubes were again weighed to observe the difference in weight after clot disruption. Finally percentage of clot lysis was determined as followings:

% of clot lysis = (wt of released clot /clot wt) × 100

RESULTS:

The aqueous and chloroform extract showed 50.1% and 62.1% respectively. Addition of 100 µl

Table: 1 Preliminary Phytochemical analysis of Methanolic , Chloroform, Aqueous extracts extract of flowers of *Bougainvillea cascade*

S. no	Test	Methanolic extract	Chloroform extracts	Aqueous extracts
1.	Tannins	+	-	+
2.	Saponins	+	-	+
3.	Flavanoids	+	+	-
4.	Terpenoids	+	+	+
5.	Glylosides	-	-	-
6.	Alkaloids	+	+	+
7.	Sterols	+	+	+

Table 2: Effect of Bougainvillea cascade flower extract of (50g/litre) on the movement of glucose out of dialysis tube over 27hr incubation period

Fractions	1h	2h	4h	6h	8h	10h	12h	24h	27h
Control	82.6±1.22	111.2±1.34	221.41±1.86	254.81±1.06	275.77±1.66	269.14±1.21	273.88±1.86	282.23±1.422	320.2±0.43
Pet ether(50g/l)	77.14±1.63*	107.31±1.21*	125.32±1.62*	137.56±1.76*	140.81±1.52*	143.77±2.13*	145.82±1.06*	149.40±1.28*	151.40±0.8*
Ethyl acetate(50g/l)	75.85±1.53*	104.44±1.56*	145.21±1.43*	156.33±1.62*	175.14±1.80*	197.77±1.78*	199.21±1.68*	209.22±1.52*	214±1.32*
chloroform(50g/l)	73.12±1.32	99.22±1.23	115.99±1.23	172.92±1.33	140.49±1.42	93.29±1.33	74.81±1.05	--	--
M ethanolic extract(50g/l)	71.15±1.23	110.5±1.03	140.49±0.23	236.66±1.25	193.3±1.23				

Values are expressed as mean + SEM of triplicate; Data were analysed using one way ANOVA followed by Tukey-Kramer multiple comparison test; *P<0.001 compared to control.

Table: 3: The comparative effect of plant extracts on haemoglobin glycosylation at various physiological glucose concentration after24, 48,72hrs of incubation

Time	standard(OD)				Ethyl acetate of B.V(OD)				Pet. Ether of B.V(OD)		
Hrs	Glucose concentration(mg/ml)				Glucose concentration(mg/ml)				Glucose concentration(mg/ml)		
	2	10	20		2	10	20		2	10	20
24	0.581	0.596	0.603		0.594	0.597	0.619		0.629	0.632	0.643
48	2.032	2.034	2.039		2.044	2.046	2.066		2.047	2.049	2.058
72	2.162	2.182	2.193		2.286	2.358	2.397		2.406	2.413	2.421

Table: 4: *In-vitro* Anti-inflammatory activities of Extracts of *Bougainvillea cascade* flowers a t various concentrations

S.no	control	Concentration (µg/ml)	Chloroform Extract of B.V(O.D)	Aqueous extract of B.V(O.D)	Standard diclofenac (O.D)
1	0.561	100	0.064±0.01	0.094±0.03	0.193±0.02
2	-	200	0.060±0.01	0.074±0.03	0.182±0.01
3	-	300	0.055±0.02	0.064±0.01	0.159±0.02
4	-	400	0.047±0.03	0.060±0.01	0.125±0.02
5	-	500	0.043±0.01	0.059±0.03	0.074±0.01

O.D-Optical density,Values are expressed as SEM of 3 readings)

Table 5: %protection of heamolysis of various Extracts of *Bougainvillea cascade* flowers

S.no	concentration (µg/ml)	Activity(%protection of heamolysis)		
		Aqueous extract of B.V	chloroform extract of B.V	Standard Diclofenac
1	Control	-	-	-
2	100	83.24	88.59	65.6
3	200	86.80	89.30	67.5
4	300	88.59	90.19	71.6
5	400	89.30	91.6	77.70
6	500	89.66	92.3	86.81

Table 6: *In vitro* thrombolytic activity of *Bougainvillea cascade* flowers extract

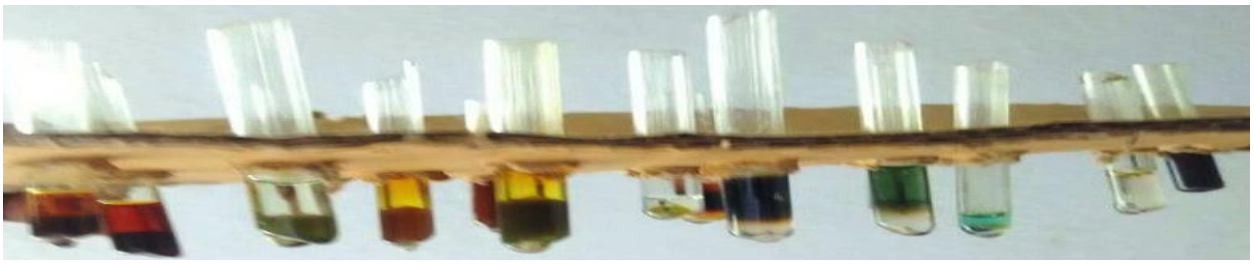
Extracts	Empty weight	Clot weight(WI)	Clot+Empty weight+ Extract weight(W2)	Clot weight after incubation (W3)	%Clot lysis W2W3/W1*100
Aqueous extract	0.94gm	0.4gm	1.4gm	1.21gm	50.1
Chloroform extract	0.92gm	0.54gm	1.55gm	1.21gm	62.1
Control	0.91gm	0.35gm	1.34gm	1.26gm	22.85
Standard	0.99gm	0.68gm	1.39gm	1.46gm	71.7

Table 7: % of clot lysis of various extracts of *Bougainvillea cascade*

S.no	**Extracts**	**% Clot lysis**
1	Aqueous extract of B.C	50.1
2	Chloroform extract of B.C	62.1
3	Control	22.85
4	standard	71.7

Figures:

Fig: 1 Preliminary Phytochemical analysis of various flower extracts of *Bougainvillea cascade*



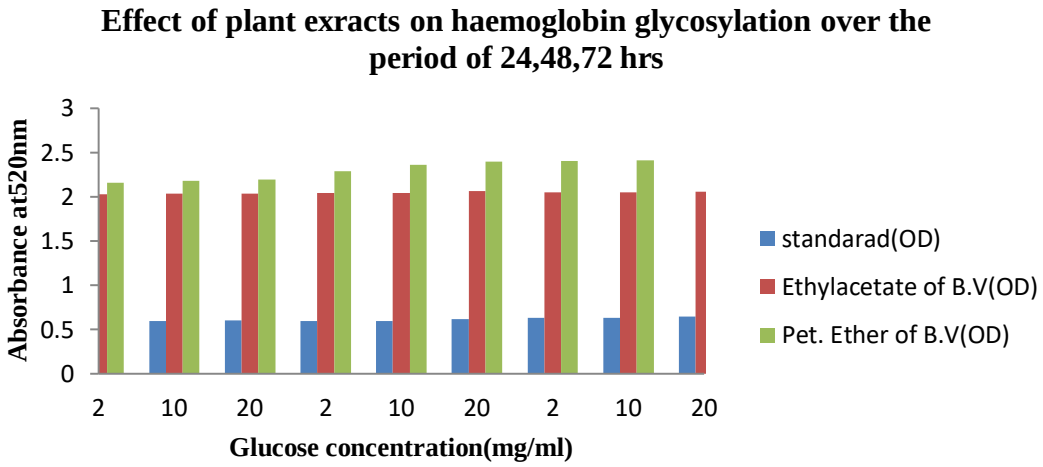


Fig: 3 **Preparation of extracts (Fig4, 5)**

Fig: 5

Fig: 6: *In-vitro* Anti-inflammatory activity of Extracts of Bougainvillea cascade flowers a t various concentrations

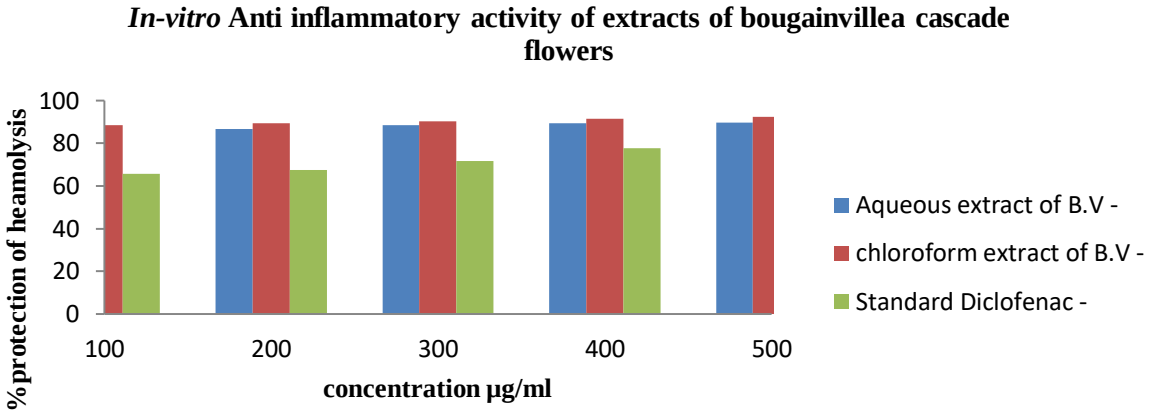
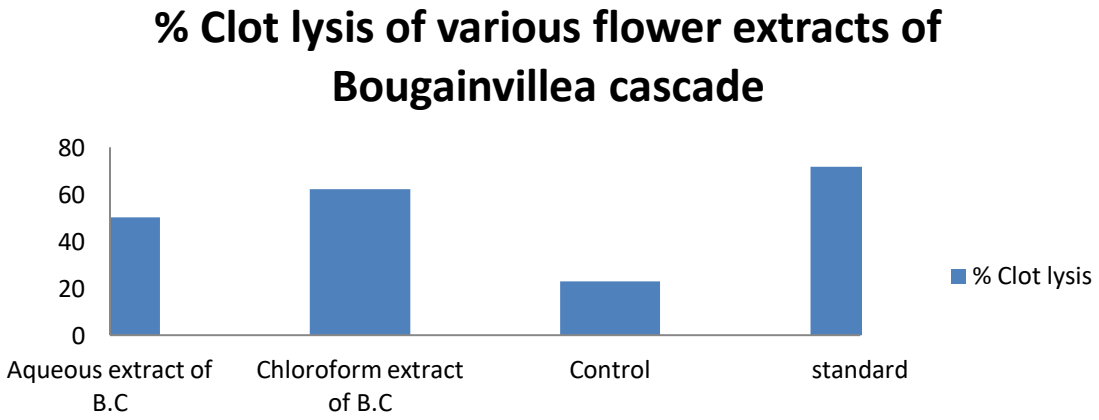


Fig: 7

Fig: 8

Fig: 9

Fig 10: In vitro thrombolytic activity of *Bougainvillea cascade* flowers extract



Streptokinase has showed 71.7% clot lysis (Table: 6, 7 & Fig 10). However, distilled water (negative control) shown only negligible clot lysis (22.85%). The mean difference in clot lysis percentage between positive and negative control was significant (p value < 0.001). The mean percentage of clot lysis by different flower extract of Bougainvillea cascade was statistically more significant when compared to those of both positive control streptokinase and negative control water.

CONCLUSION:

Through our study it was found that aqueous and chloroform extracts of Bougainvillea cascade possesses thrombolytic activity[20]. By the above obtained results, it can be suggested that the application of the Bougainvillea cascade component may be accessible for greater section of the society for the treatment of cardiovascular diseases. In addition, positive result in thrombolytic activity test led us to the interference that the plant extract may contain bioactive compounds, which may aid ongoing cardiovascular drug discovery from the floristic resources. Hence, further studies are suggested to be undertaken to pin point the exact compounds and to better, understand its actions.

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