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PRELIMINARY PHYTOCHEMICAL SCREENING AND EVALUATION OF INVITRO ANTISNAKE VENOM ACTIVITY OF *ALANGIUM SALVIFOLIUM*

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Abstract

Alangium salvifolium, traditionally used in the treatment of the various diseases. Almost every part of *A. salvifolium* including roots, leaves, stem and bark were used in the Ayurveda and Siddha systems of medicines for the treatment of various diseases. In modern scientific literatures, the plant has been reported to have potential efficacy against diabetes, peptic ulcer, arthritis, inflammation and anthelminthic activities etc. *A. salvifolium* is reported to contain various biologically active phytochemicals such as alangine, ankorine, tubulosine, alangicine, salsoline etc. The present study highlights the pharmacognostic, phytochemical and in vitro anti-snake venom evaluation of *A. salvifolium*. This research focused on the investigating the most effective successive extract showing anti-snake venom activity through evaluating the phospholipase A2 (PLA2) activity of Russell's viper venom. Platelet aggregation induced by the different root extract of *A. salvifolium* was evaluated by taking the two dose i.e. 250 and 500 mcg/ml and it was found that 500 mcg/ml of methanolic extract shows maximum platelet aggregation (79.99 %). The effect of each successive root extract of *A. salvifolium* on Russell's viper venom (PLA2) induced haemolysis on sheep RBC's gel plate was also evaluated to confirm the anti-snake venom activity and was found the same extract i.e. methanolic successive extract having dose of 300 and 600 mcg had shown the maximum inhibition as compared to other extract.

Keywords: *Alangium salvifolium, Anti-snake venom, Preliminary pharmacognostic, Russell's viper venom*

Introduction

Alangium salvifolium Wang is a deciduous, climbing shrub or a tree belonging to the family Alangiaceae. Different parts of this plant are used for a wide range of diseases. Root bark is emetic, febrifuge, purgative, anthelmintic, diaphoretic, antipyretic and useful in fever, snake bite and piles. Its root is used as diuretic, astringent and antidote for several poisons [1]. Literature surveys revealed [2, 3, 4, 5] that very few chemical and biological studies have been done on roots of *A. salvifolium* but there is no specific study on successive extracts of the same.

Snakebite leads to so many deaths worldwide. It is a major issue, especially in tropical countries like India. It has been estimated that 5 million people are bitten by venomous snakes annually around the world, there by resulting in about 100,000 fatalities [6, 7]. In India more than 200,000 cases are reported and an estimated 35,000 to 50,000 people die each year [8]. From the literature review it was found that the plant *Alangium salvifolium* claimed to be useful in treating snake poison in the traditional system of medicine. As this plant has not been proven scientifically for its antisnake venom activity earlier, therefore the present study is the first attempt to evaluate the pharmacognostics parameters, chemical entity and especially anti-venom activity of successive extracts of roots of *A. salvifolium*, which contains petroleum ether, benzene, chloroform, ethyl acetate, methanol and water extract in order to understand the secondary metabolites responsible for the biological activity.

Methods

Collection and authentication of plant: Roots of the plants *Alangium salvifolium* were collected from Munjaka, kalawad road, Rajkot and was authentified by NISCAIR, New Delhi. For the future reference drive, the herbarium was stored S. J. Thakkar Pharmacy College, Rajkot.

Plant powder preparation: The roots *A. salvifolium* were cut in the form of thin-round slices and sun dried. The dried plant material was after imperiled to coarse powder and passed it from sieve no. 40 #. The prepared powder was preserved into the airtight container for future proceeding. Morphological evaluation and microscopical evaluation of *A. salvifolium* were done.

Physicochemical evaluation [9]: Ash value (Total ash, Acid insoluble, Water soluble, Sulphated ash and Nitrated ash), extractive value (Water & alcohol soluble extractive), Foreign organic matter, Loss on drying, Total phenolic content, Heamolytic index and Foaming index were performed for the study of physicochemical evaluation of *A. salvifolium*.

Phytochemical Analysis/Screening [10]: The roots of *A. salvifolium* were successively extracted with various solvents viz. petroleum ether, chloroform, acetone, methanol and water. Presence of alkaloids, glycosides, carbohydrates, phenolic compounds and tannin etc were qualitatively checked into the obtained extract through chemical test.

Thin Layer Chromatography (TLC) Profiling or Fingerprinting [11]: TLC profile was performed for each successive root extracts of *A. salvifolium.* The selected mobile phases for each successive extracts, which developed on the trial and error basis was listed in table 1. The developed plates were observed under UV light 254, UV 366 and visible 540. Afterwards, the development was carried out by using different spraying reagent.

Anti-snake venom activity: Platelet aggregation and phospholipase inhibition are the parameters to evaluate the preliminary *in-vitro* anti-snake venom activity. The same activity was studied on the successive extract of *A. salvifolium* by evaluating the mentioned parameters to select the most active promising extracts.

Venom collection and preservation: The lyophilized snake venom *Viper russelli* was obtained from Irula Snake Catcher's I.C.S. Ltd., Vadanemmeli Village, Kancheepuram Dist., Tamil Nadu, India and was preserved at 4° C. For the anti-snake venom activity, preserved venom was dissolved in saline, centrifuged at 2000 rpm for 10 min and the supernatant used for the same.

a) ADP induced platelet aggregation [12]: Snake venom contains several isospecific phospholipase A_2 (PAL A_2). Snake venom PAL A_2 are responsible for the presynaptic or postsynaptic neurotoxicity, myotoxicity, cardiotoxicity, inhibition of platelet aggregation, haemolytic, convulsant, hypotensive, anticoagulant activity etc.

ADP induced platelet aggregation in platelet rich plasma was inhibited in the presence of phospholipase A_2 which was present in viper venom. The test drug reacts with PAL A_2 enzyme and there by decreases the inhibition of platelet aggregation.

The reaction mixture contains different concentration of drug extract 0.5 ml (250, 500 µg/ml), venom solution 0.5 ml (200 µg/ml) and platelet rich plasma 0.5 ml. These reaction mixtures was maintained at 37°C and kept for 2 min with constant stirring, 0.5 ml of ADP solution was added and incubated for 4 mins and absorbance was measured at 414 nm.

$$\% \text{ ADP induced platelet aggregation} = \frac{(T_1-T_2)-(T_1-T_3)}{(T_1-T_2)} \times 100$$

Where,

$T_1 \leftrightarrow$ ADP + Platelet

$T_2 \leftrightarrow$ ADP +Venom +Platelet

$T_3 \leftrightarrow$ ADP +Venom +Platelet +Plant extract

b) Determination of Neutralization of Phospholipase A_2 activity [13, 14] :

i) Determination of MHD of Russell's viper venom: For evaluating the phospholipase A2 (PLA_2) activity of Russell's viper venom, the indirect haemolytic activity was assayed. 300 µl of packed sheep erythrocytes washed four times with saline solution, 300 µl of 1:3 egg yolk solution in saline solution and 250 µl of 0.01 M $CaCl_2$ solution were added to 25 ml of 1% (w/v) of agar at 50°C & dissolved in PBS pH 7.2. The mixture was applied to Petri dish and allowed to gel. Then, 3 mm diameter wells were made and filled with 15 µl venom samples. After 20 hr of incubation at 37°C, the diameters of haemolytic halos were measured. To determine the minimum haemolytic dose (MHD) of Russell's viper venom, 15 µl of solutions containing different concentrations of venom (from 1 to 50 µg) were applied into the wells. Control wells were containing 15 µl of Phosphate buffer of pH 7.2. After 20 h of incubation at 37°C, the diameters of haemolytic halos were measured. The minimum haemolytic dose (MHD) was defined as the amount of venom that induced a haemolytic halo of 11-mm diameter.

ii) Neutralization of Phospholipase activity (MHD): Each successive root extracts were evaluated for neutralization of phospholipase A2 activity (indirect haemolytic activity). Samples of constant amount of venom (1 MHD) were incubated with different amount of Extract (150, 300 and 600 µg) for 30 min at 37 °C. Then aliquots of 15µl of the mixtures (venom + Extract) was added to wells in agarose-egg yolk-sheep-erythrocytes gel plates and incubated at 37 °C for 20 hr. Control samples were contained venom (1 MHD) without extract. Plates were incubated at 37 °C for 20 hr. Neutralization is expressed as the % Inhibition that reduced 50% the diameter of the haemolytic halo when compared to the effect induced by venom alone.

Statistical analysis: All data are presented as mean ± standard deviation (SD). Data were evaluated by one-way analysis of variance (ANOVA) using SPSS Version 15.0 (SPSS Inc., Chicago, IL, USA). The level of significance was set at $p < 0.05$ for all statistical tests.

Results

Microscopically evaluation: TS of the root shows outer cork, broad irregular layers of cork showing development of rhytidome, a narrow band of cortex, phloem and xylem (Figure 1). Cork is well developed. Cork consists of 5 or more successive layers with alternating parenchymatous tissue in between them due to the formation of phellogen at different levels in the outer phloem region of the root. The phloem present from the cambium up to the cortex. The cells are thin-walled and regularly arranged except that the cells towards the cortex are larger in size.

The phloem elements are arranged in narrow strips with alternating medullary rays. Sieve elements and companion cells are separate. The phloem parenchyma cells contain calcium crystals. Most of these cells of the phloem are filled with simple round starch grains. Cambium is a narrow consist of 4-5 rows of properly arranged thin-walled cells. The xylem is centrally located

and is lignified. It consists of xylem elements such as parenchyma, vessels and fibres. Xylem and phloem are separated by cambium.

Microscopy of powder of roots of *A. salvifolium*: Microscopic examination of the powder showed xylem vessels, xylem parenchyma, cluster crystals of calcium oxalate, cork cells, 2-3 layers medullary rays and thin-walled phloem parenchyma with crystals of calcium oxalate,.

Physicochemical parameters of Roots of *A. salvifolium*: The different physical parameters obtained were listed in Table no.2.

Qualitative chemical tests of successive root extracts of *A. Salvifolium:* Each successive extract was used to perform various phytochemical tests. It was shown in Table no.3.

TLC fingerprinting of successive extracts of *A. salvifolium:* TLC study of each successive extract revealed the presence of various phytoconstituents. TLC plates were observed under short and long wavelength as well as sprayed with various spraying reagent. The results were shown in Figure 2, 3, 4 and 5.

Evaluation of anti-snake venom activity of successive extracts of *A. salvifolium*

a) ADP induced platelet aggregation: The PAL A2 present in the viper venom causes inhibition of ADP induced platelet aggregation. All successive root extracts were significantly capable of decreased the inhibition of platelet aggregation in a dose dependent manner as shown in table 4.

b) Determination of Neutralization of Phospholipase A_2 activity

i) Determination of MHD of Russell's viper venom: In phospholipase A_2 activity, Russell's viper venom able to produce haemolytic haloes in agarose-sheep erythrocytes gels (table 5). About 12 µg of Russell's viper venom produced 12 mm diameter haemolytic halo. This shows that Russell's viper venoms have the enzymes (phospholipase A_2) that has the ability to lyse sheep RBC's.

Discussion

Petroleum ether and Benzene extract shows the presences of phytosterol/terpenoids while alkaloids and Phytosterols/Terpenoids both were present into the Chloroform extract. Acetone shows the presence of alkaloids, phenolic compounds and tannins while alkaloids, carbohydrates, Phenolic compounds, tannins and flavonoids were present into the ethanol extracts. Water extract contains carbohydrates, Saponins, phenolic compounds, tannins and flavonoid.

The TLC of Petroleum ether, benzene and chloroform extract showed presence of steroidal and terpenoidal compounds with different Rf value. The plates were sprayed with Libermann Burchard Reagent showed blue to violet spot indicates the presence of various phytosterols. The TLC of chloroform, ethyl acetate and methanolic extract were sprayed with Dragendroff's reagent showed orange spots indicates the presence of alkaloids. The TLC of Ethyl acetate, Methanol and water extract were sprayed with ferric chloride and anisaldehyde reagent showed blue, pink and violet spots at different Rf value. The above results showed the presence of various Phenolics and flavanoids.

As compare to each successive root extract methanolic extract showed better platelet aggregation and inhibition of phospholipase A_2. The Methanolic root extract of *A. salvifolium* showed a maximum of 79.99 % ADP induced platelet aggregation at a concentration of 500µg/ml. 600µg/ml of Methanolic extract of plants was able to produce more than 85% inhibition of halos produced by the phospholipase A_2 dependent haemolysis of sheep RBC's induced by Russell's viper venom. These results suggested that protective action of Methanolic extract of plants against Russell's viper venom due to inhibition of mainly PLA_2 enzyme of venom. In contrast, saline solution did not induce haemolysis. When egg yolk is not added to the gels there was no haemolysis, indicating that haemolysis was only of the indirect type, i.e. due to PLA_2 activity in this venom.

Conclusion

Present study revealed that *A. salvifoliu*m roots have significant antisnake venom activity. Methanolic extract showed better anti-venom activity as compared to other successive extracts. Hence, these findings support the fact that this plant could be useful in herbal healthcare system against snake bite. However further studies are needed to isolate and characterize the active

constituents responsible for antisnake venom activity.

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Table 1: Selected mobile phase for different successive root extract of *A. sa*lvifolium

Sr. No.	Name of extract	Mobile phase
1	Pet. Ether extract	Mobile phase: Pet. Ether: Ethyl acetate (4:1)
2	Benzene extract	Pet. Ether: Ethyl acetate (4:1)
3	Chloroform extract	Chloroform: Methanol (4:1)
4	Ethyl acetate extract	Chloroform: Ethyl acetate (3:2)
5	Methanol extract	Chloroform: Methanol: Formic Acid (2.5:2:0.5)
6	Water extract	Chloroform: Methanol (4.5:0.5)

Table 2: Physicochemical parameters of Roots of *A. salvifolium*

Sr. no.	Physical Parameters	Average values %w/w
		Roots of *A. Salvifolium*
1	Total ash	4.87 ± 0.76
2	Acid insoluble ash	1.57 ± 0.12
3	Water soluble ash	2.09 ± 0.18
4	Sulphated ash	2.56 ± 0.08
5	Nitrated ash	1.83 ± 0.34
6	Carbonated ash	1.03 ± 0.23
7	Water soluble extractive	9.35 ± 0.13
8	Alcohol soluble extractive	6.98 ± 0.76
9	Loss on drying	11.34 ± 0.38
10	Hemolytic activity	Nil
11	Foaming index	>100
12	Total Phenolic content	22.56 ± 0.15mg/gdw*

Table 3: Qualitative chemical tests of successive root extracts of *A. salvifolium*

Sr. No.	TEST	P. E.	C_6H_6	$CHCl_3$	Acetone	Ethanol	H_2O
1)	Alkaloids	-	-	+	+	+	-
2)	Carbohydrates	-	-	-	-	+	+
3)	Phytosterols/Terpenoids	+	+	+	-	-	-
4)	Fixed oils and fats	-	-	-	-	-	-
5)	Saponins	-	-	-	-	-	+
6)	Phenolic comp. & tannins	-	-	-	+	+	+
7)	Proteins & amino acids	-	-	-	-	-	-
8)	Gums and mucilage	-	-	-	-	-	-
9)	Volatile oil	-	-	-	-	-	-
10)	Flavonoid	-	-	-	-	+	+

Table 4: Platelet aggregation induced by the different root extract of *A. salvifolium*

Sr. No.	Drug	Conc. µg/ml	% Platelet aggregation
1	T_1		
2	T_2		
3	Petroleum ether ext	250	5.46 ± 0.18
		500	12.91 ± 0.04
4	Benzene extract	250	17.68 ± 0.39
		500	27.64 ± 0.23
5	Chloroform extract	250	19.54 ± 0.1
		500	37.77 ± 0.64
6	Ethyl acetate extract	250	33.14 ± 0.09
		500	50.35 ± 0.83
7	Methanol extract	250	65.22 ± 0.54
		500	79.99 ± 0.14
8	Water extract	250	55.92 ± 0.38
		500	65.59 ± 0.18

*p(0.03) < 0.05, so statically there is significant difference

Table 5: The effect of Russell's viper venom (PLA_2) induced haemolysis on sheep RBC's gel plate

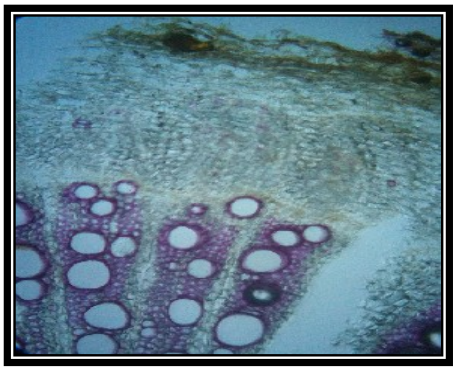
Venom (µg)	Haemolytic halos (mm)
4	3.3 ± 0.04
8	9.1 ± 0.35
12	12 ± 0.02
16	15.2 ± 0.23
20	19.6 ± 0.19

Table 6: The effect of each successive root extract of *A. salvifolium* on Russell's viper venom (PLA_2) induced haemolysis on sheep RBC's gel plate

Root extract	Venom (12 µg) + Extract (µg)	Haemolytic halos (mm)	% Inhibition
Petroleum ether	150	11.73 ±0.24	2.25
	300	11.42 ± 0.3	4.83
	600	10.8 ± 0.72	10
Benzene	150	10.8 ± 0.24	10
	300	10.7 ± 0.24	10.83
	600	9.5 ± 0.16	20.83
Chloroform	150	9.9 ±0.12	17.5
	300	9.3 ±0 .43	22.5
	600	6.5 ± 0.4	45.83
Ethyl acetate	150	8.9 ± 0.23	25.83
	300	7.3 ± 0.11	39.17
	600	5.3 ± 0.7	55.83
Methanol	150	6.9 ± 0.13	42.5
	300	4.6 ± 0.19	61.67
	600	1.9 ± 0.45	84.17
Water	150	7.4 ± 0.28	38.33
	300	5.8 ± 0.16	51.67
	600	4.2 ±0.14	65

*p(0.03) < 0.05, so statically there is significant difference

Figure 1: Microscopy of root of *A. salvifolium*



1- Pet. Ether, 2- Benzene, 3-Chloroform, 4-Ethyal acetate, 5-Methanol extract

Figure 2: TLC plates of different extract of *A. salvifolium* under UV 254

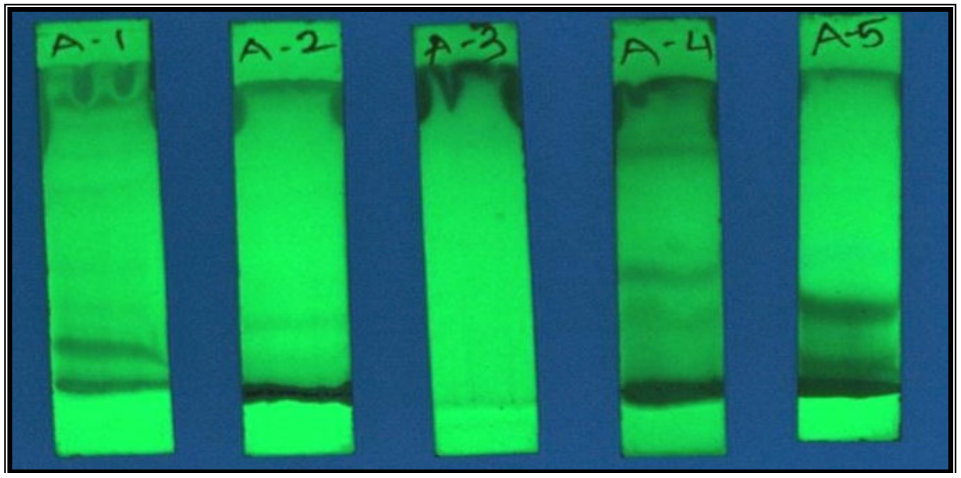


Figure 3: TLC plates of different extract of *A. salvifolium* under UV 366

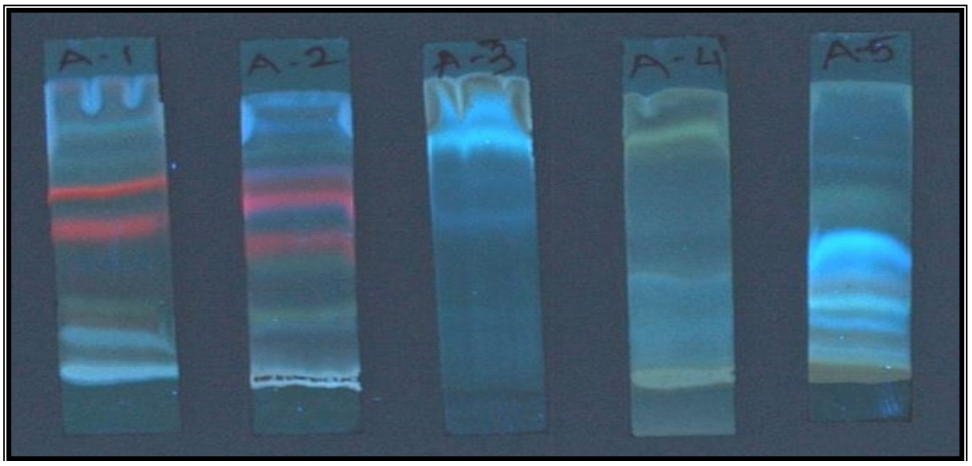


Figure 4: TLC plates of different extract of *A. salvifolium* under Visible 540

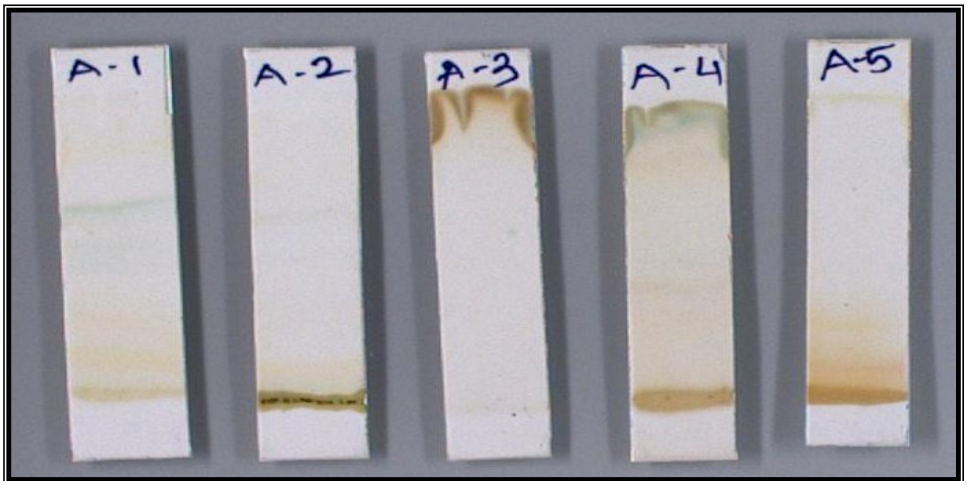


Figure 5: TLC plates of different extract of *A.salvifolium* after development with various spraying reagent

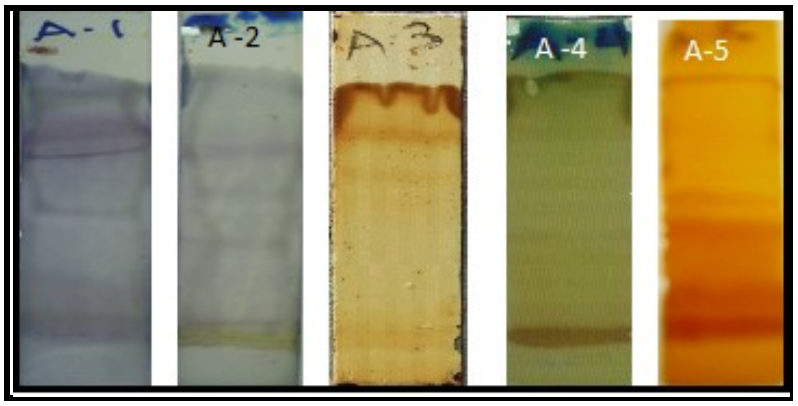


Figure 6: Platelet aggregation induced by the different root extract of *A. salvifolium*

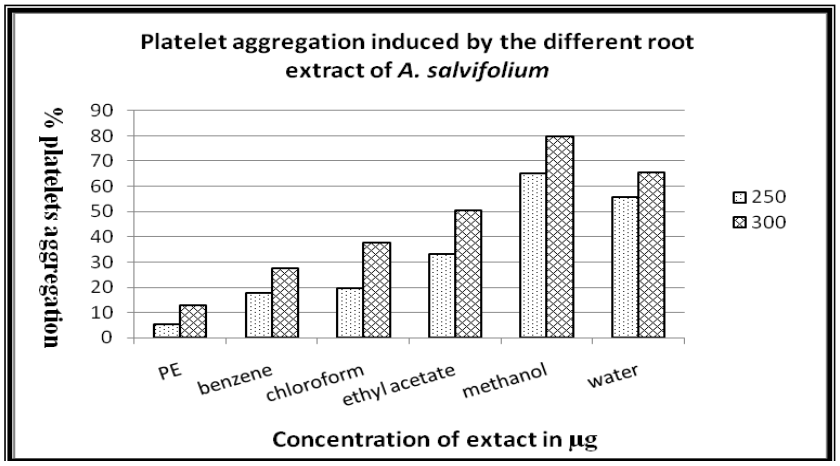


Figure 7: Inhibiting activity of PLA_2 by the different root extract of *A. salvifolium*