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Dr. R Malathi
Research Department of Biotechnology, Bharathidasan University Constituent College, Perambalur, Tamil Nadu, India

D Sivakumar
Research Department of Biotechnology, Bharathidasan University Constituent College, Perambalur, Tamil Nadu, India

S Chandrasekar
Research Department of Biotechnology, Bharathidasan University Constituent College, Perambalur, Tamil Nadu, India

Corresponding Author
Dr. R Malathi
Research Department of Biotechnology, Bharathidasan University Constituent College, Perambalur, Tamil Nadu, India

Studies on pharmacological and biochemical effects of Indian saw scaled viper venom and its inhibition by *Acalypha fruticosa* leaves extract

Dr. R Malathi, D Sivakumar and S Chandrasekar

Abstract
Snakebite is a significant cause of death and disability in subsistent farming populations of rural India. Though antisnake venom serum is the only therapeutic remedy available for treating the victims till date, much scientific attention has also been given to the identification and isolation of plant derived principles for the treatment of snakebite victims during last two decades. *E. carinatus* bite is a serious threat to South Asian countries including India, as it causes the highest number of deaths and debilitating sustained tissue necrosis at the bite site. *Acalypha fruticosa* (*Euphorbiaceae*), one such shrubby plant species, generally distributed in the southern Western Ghats of India upto 1800m above mean sea level is traditionally prescribed by the local medical practitioners in the western districts of Tamil Nadu, India (Villupuram, Coimbatore, Tirupur, Erode and Dindugal) for various ailments. The leaves are prescribed for digestive troubles such as dyspepsia, colic and diarrhea and even to treat cholera. Leaves are used to treat burns and often used for venomous bites and stings. In the present study, the ethanolic extract of *A. fruticosa* leaves was used to evaluate the antisnake venom activity against the Indian saw scaled viper venom. The ethanolic extract was effectively inhibited the enzyme activity that present in Indian saw scaled viper venom which play a major role in the lethal effects.

Keywords: *Acalypha fruticosa,* Indian saw scaled viper, enzyme inhibition, 5'nucleotidase

1. Introduction
The venomous snakebite affects more than millions of livings annually in worldwide, the major portion of these affected beings are from underdeveloped and developing nations from Asia, Africa, and Latin America. The yearly epidemiology data have been recorded to about 1.8 million venomous bites and stings leading to about 0.125 million deaths. The snake bite envenoming comprises a significant public health problem with serious medical consequences that mainly affects rural farming populations, especially in tropical and subtropical regions. It is a provincial, environmental and an occupational bio accident, rather than it is known as a 'Neglected Tropical Disease' (Slagboom *et al.,* 2017) [14].

Venom is a cocktail of actively pharmacological proteins and peptides as well as low molecular weight compounds including lipids, carbohydrates and amines that target several tissues induce pain in the bitten site and different systemic disorders (Laraba Djebari and Cherifi, 2014) [17]. Viper venom is mostly rich source of metalloproteases and serine proteases which interfere with the physiological processes of victim (Fahmi *et al.,* 2016) [9].

There are 52 poisonous snakes in India, among them only 4 are considered as highly venomous and responsible for more number of deaths. The four poisonous snakes namely Indian cobra (*Najanaja*), Saw scaled viper (*Echis carinatus*), Russell's viper (*Daboia russelli*) and common Krait (*Bangarus caeruleus*) found in India are responsible for the highest death rates among the rural populations of India (Gomes *et al.,* 2010) [11].

Echis carinatus is a highly medically important snake in India. Saw scaled viper are one of the highly venomous snake found in Indian subcontinent and its venom has a wide variety of proteins, enzymes and peptides and it can able to causing severe envenoming characterized by incoagulable blood and potentially severe systemic bleeding in victim (Warrell *et al.,* 2013) [29]. Saw scaled viper belonging to the *Viperidae* family and it is thought to be responsible for more snakebite death in worldwide than any other snake species. Its venom contains the protein that affect the transformation of prothrombin into thrombin. Saw scaled viper bite mainly associated with severe local toxicity, coagulopathy, hemorrhage, myotoxicity, rapid progression of local edema and active bleeding (Fonseka *et al.,* 2013) [10].

In India, traditional populations approaches to traditional healers for the treatment of venomous snakebites and it is considered as most reliable and acceptable practice for the snakebite treatment. In rural places, herbal medicines for the snakebite treatment are readily

readily available and so many ethnic groups of the villages administered the herbal medicines without prior to the antiserum administration and saved lives of snakebite victims (Gomes *et al.*, 2010) [11].

Acalypha is considered as the fourth largest genus among the *Euphorbiaceae* family. It consists of 450 species, in the form of evergreen shrubs, trees and annuals. *Acalypha fruticosa* belongs to the family *Euphorbiaceae* commonly known as Sinni maram in Tamil and Birch leaved Acalypha in English, is widely used in traditional medicine for the treatment of various ailments. In folklore remedy the plant was used in the treatment of cancer among the tribal population in Kolli Hills, South India. However, fewer reports are available with respect to the pharmacological properties of the plant (Sripathi and Uma, 2010; Alasbahi *et al.*, 1999) [27, 2]. To the best of our knowledge, there are no any previous studies conducted to evaluate the antisnake venom activity of *Acalypha fruticosa* leaves against the Indian saw scaled viper venom. Hence we selected this plant for this purpose.

2. Materials and Methods

2.1 Sample collection

The fresh plant leaves of *Acalypha fruticosa* was collected in the month of November - December in 2017 at the village of Thenkuchipalayam, Villupuram district, Tamilnadu state, India (11.8634° N, 79.5168° E). Then the leaves was washed under running tap water for remove the dust matters and unwanted contaminants. Then the leaves were shade dried and powdered using mechanical grinder. The fine powder was stored in airtight container.

2.2 Extract preparation

The extract was prepared by both hot and cold percolation methods. 20g of dried and powdered plant leaf was soaked in 100 ml of acetone, chloroform and water respectively. Powder with acetone and chloroform solvents were kept at room temperature by occasional shacking for 48hrs (Sharmistha Chakravarthy and Chandra Kalita Jogen, 2012) [26]. The water was boiled for over 1 hour at 100˚C. Then the extracts were filtered by using Whatmann No.1 filter paper. Then the extracts was stored in an air tight container then refrigerated at 4˚C for further use.

2.3 Qualitative phytochemical studies

The individual extracts were subjected to different qualitative chemical investigation for the establishing profile of the given extracts for their chemical composition (Raaman, 2006) [24]. The crude powder was extracted in different solvents are tested for various phytoconstituents present in them by standard procedure **(**Harborne, 1973; Kokate, 1997) [13, 16]. They are generally tested for the presence of alkaloids, flavonoids, tannins, phenols, cardiac glycosides, triterpenes, steroids and saponins.

2.4 Collection of venom

The crude venom was extracted from the medium sized Indian saw scaled (*Echis carinatus*) viper snake capture by the Irula tribal of Kurumbalur village, Perambalur district. The extracted crude venom was dissolved in phosphate buffer and centrifuged at 2000 rpm for ten minutes. The supernatant was used for further analysis and stored at 4˚C.

2.5 *In vitro* snake venom inhibition studies

2.5.1 Inhibition of 5'Nucleotidase enzyme activity

5′ Nucleotidase was assayed by the method of Rowe *et al.* 1980 [25]. The substrate solution contained 1 mL of Tris–HCl buffer (pH 8.0), 0.1 mL of 0.1 M magnesium chloride and 0.8 mL of 0.15% 5′AMP followed by 0.25 mL of 0.1% crude venom and incubated at 37°C for 15 minutes. At the end of incubation time, the reaction was quenched by adding TCA and filtered. The filtrate was assayed for inorganic phosphate at 625 nm using potassium dihydrogen phosphate as standard. In this analysis, one unit of enzyme activity was defined as the amount that yielded 0.01 µmole of inorganic phosphate/minute under the experimental conditions. For inhibition studies, the venom was preincubated with the various concentrations of (50µg to 400µg) plant extract for 30 minutes at 37°C.

2.5.2 Inhibition of Phospholipase A_2 enzyme activity

A reaction mixture containing 0.5 ml of lecithin (50mg dissolved in 25ml of diethyl ether), 1ml of venom solution (0.04%) in various concentrations (0.5ml to 2.5 ml), and 0.1ml of 5% of $CaCl_2$ solution were added and the reaction vessel was swirled or shaken until the reaction mixture becomes homogenous. The reaction was allowed to proceed under room temperature for 4 hours. At the end of incubation period 25ml of alcohol and 0.3ml of cresol red solution were added and the solution titrated with 0.02N methanolic NaOH. A blank titration was performed was prepared by addition of alcohol, venom and $CaCl_2$ to the ether in that order and was titrated immediately. The hydrolysis capacity of PLA_2 exhibited by 400µg/ml of venom with 50mg lecithin was considered as 100% phospholipase activity and served as control. For inhibition studies, the venom was preincubated with the various concentrations of (50µg to 400µg) plant extract for 30 minutes at 37°C.

2.5.3 Inhibition of Phosphomonoesterase activity

The phosphomonoesterase activity was to be determined by the method of Bessey *et al.*, (1946) [4] with slight modifications. The reaction mixture included 1 ml of Tris–HCl buffer (pH 8.0), 1.0 ml of 0.0025M sodium-p-nitrophenol phosphate, various concentration (0.1 ml to 0.5ml) of 0.25% crude venom and was added to together and incubated at 37˚C for three hours. The absorbance was measured in UV visible spectrophotometer at 425 nm. p-Nitrophenol was used as the standard. One unit of enzyme activity was defined as the amount that yielded0.1 µM of p-nitrophenol/hour under the experimental conditions. For inhibition studies, the venom was preincubated with the various concentrations of (50µg to 400µg) plant extract for 30 minutes at 37˚C.

2.5.4 Inhibition of Phosphodiesterase enzyme activity

Phosphodiesterase activity was to be determined by a method modified from Lo *et al.*, (1966) [19]. The assay mixture contained various concentration of (0.1ml to 0.5ml) venom solution, 0.5 mL of 0.0025 M Sodium-p-nitrophenyl phosphate, 0.3 mL of 0.01M $MgSO_4$ and 0.5 mL of 0.17M Tris–HCl (pH 8.0) and was incubated at 37°C for three hours. The absorbance was measured at 400 nm. Phosphodiesterase activity was expressed in nanomoles of product released/minute. The molar extinction coefficient at 400 nm was 8100 Cm^{-1} M^{-1}. For inhibition studies, the venom was preincubated with the various concentrations of (50µg to 400µg) plant extract for 30 minutes at 37˚C.

2.5.5 Inhibition of L-Amino acid oxidase

The L-amino acid oxidase activity was to be carried out according to Li *et al.*, (1994) [18]. The reaction mixture consisted of 1.0 mL of 0.1% L-leucine, 2.0 mL of Tris–HCl buffer (pH 8.0), 0.25 mL of 0.1% dianisidine hydrochloride,

0.15 mL of 0.1% horseradish peroxidase and various concentrations (0.04 ml to 0.18 ml) of 0.5% crude venom solution. It was allowed to stand for ten minutes at room temperature and then the absorbance was measured at 415 nm. One unit (U) was defined as the amount of enzyme that catalyzed the formation of 1 µmol H_2O_2 per minute. For inhibition studies, the venom was preincubated with the various concentrations of (50µg to 400µg) plant extract for 30 minutes at 37˚C.

2.5.6 Inhibition of Acetylcholine esterase enzyme activity

Acetylcholine esterase activity was to be assayed by the following Ellman *et al.,* (1961) [8] method. The reaction mixture comprised 3.0 mL of the phosphate buffer (pH 8.0), 10 µL of DTNB (10 mmole/L) and 20 µL of acetylethiocholine iodide (158.5 mmol/L). A total of 50 µL of 0.1% crude venom and 3 mL of buffer solution were incubated at room temperature for five minutes. Then, 10 µL of DTNB (a strong oxidizing agent) and 20 µL of substrate acetylethiocholine iodide were added in order to reach a final concentration of 1 mmole/L. The increase in absorbance at 412 nm was measured on a double beam spectrophotometer against control mixture prepared at the same time. However, in the latter case, 50 µL of enzyme was replaced with 50 µL of buffer solution. For inhibition studies, the venom was preincubated with the various concentrations of (50µg to 400µg) plant extract for 30 minutes at 37˚C.

2.5.7 Inhibition of Hyaluronidase enzyme activity

The hyaluronidase assay of crude venom was to be determined turbidometrically by the method of Pukrittayakamee *et al.,* (1988) [23]. The assay mixture contained buffer Tris–HCl (pH 8.0), 0.5 ml of hyaluronic acid (0.5 mg/mL in buffer) and various concentrations (0.05 ml to 0.25 ml) of venom solution. The mixture was incubated for 15 minutes at 37˚C and the reaction was quenched by the addition of 2 mL of 2.5% (w/v) cetyltrimethylammonium bromide in 2% NaOH (w/v). The absorbance was read at 400 nm (within ten minutes) against a control solution containing 1 mL of the same buffer and 2 mL of 2.5% (w/v) cetyltrimethylammonium bromide in 2% NaOH (w/v). Turbidity reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of a tube in which no enzyme was added as 100%. One unit was defined as the amount of enzyme that provoked 50% turbidity reduction. Specific activity was defined as turbidity reducing units per milligram of enzyme. For inhibition studies, the venom was preincubated with the various concentrations of (50µg to 400µg) plant extract for 30 minutes at 37˚C.

2.5.8 Inhibition of protease enzyme activity

Protease assay of crude venom was performed according to the method of Greenberg, (1955) [12]. The reaction mixture composed of 1 ml of 0.5% casein, 1.0 mL of Tris–HCl buffer (pH 8.0), 0.5 mL of 0.25% crude venom and the reaction mixture incubated for four hours at 37°C. At the end of four hours, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA) and filtered. The filtrate was used for protein estimation by the method of Bradford methodusing L-tyrosine as a standard. In the above investigation, one unit of enzyme activity was defined as the amount that yielded 0.02 µmole of tyrosine/hour under experimental conditions described. For the inhibition studies, venom was preincubated with the extracts for 30 minutes at 37° C.

2.6 Statistical analysis

Results obtained were reported as mean ± SD of triplicate measurements. Significance differences for multiple comparisons were determined by One-way ANOVA with $p<0.005$ using SPSS (version 19).

3. Results

3.1 Phytochemical analysis result

The present study has been revealed that the presence of phytochemicals considered as active medicinal chemical constituents. Important medicinal phytochemicals such as flavonoids, carbohydrates, cardiac glycosides, proteins, xantho protein, phenols, fatty acids, phlobatannins and emodins were present in the samples. The result of the phytochemical analysis shows that the plant *Acalypha fruticosa* are rich in at least one of the flavonoids, protein, xantho protein, phenols, carbohydrates, cardiac glycosides, fatty acids, phlobatannins and emodins. The results of phytochemical analysis (Table 1) was clearly indicates that the ethanolic extract showed the presence of highest number of phytochemicals. Hence the ethanolic extract was selected for snake venom enzyme inhibition studies.

Table 1: Results of qualitative phytochemical analysis of *Acalypha fruticosa* leaf extracts

S. No	Name of the compounds	Name of the solvents				
		Diethyl ether	Ethanol	Ethyl acetate	Methanol	Petroleum ether
1	Alkaloids	-	++	++	+	+
2	Flavonoids	++	++	-	+	++
3	Carbohydrates	-	++	+	+	-
4	Glycosides	-	+++	+++	+++	++
5	Cardiac glycosides	-	+	+	+	-
6	Coumarins	+	+	-	-	+
7	Saponins	-	+	+	+	+
8	Hydroxy anthraquinones	-	++	-	+	-
9	Tannins	-	+	-	+	+
10	Phlobatannins	-	+	-	-	-
11	Proteins	++	++	+	+	++
12	Xantho protein	+	+	+	+	+
13	Amino acids	-	-	-	-	-
14	Steroids	-	+	-	-	-
15	Terpenoids	+	+	+	+	+
16	Phenols	-	++	+	+	+
17	Resins	-	+	+	+	+
18	Volatile oil	-	+	+	-	+
19	Fatty acid	-	-	+	+	-
20	Emodins	-	+	-	-	-

+ → present in small concentration; ++ → present in moderately high concentration;
+++ → present in very high concentration; -- → absent or negative result

3.2 Results of enzyme inhibition studies

The ethanolic extract of *Acalypha fruticosa* leaves showed the significant ($p<0.005$) enzyme inhibitory activity against the Indian saw scaled viper venom. Enzymes are the major components of the snake venom and it is responsible for the lethality. In the present study, the ethanolic extract of *Acalypha fruticosa* leaves was tested against 8 enzymes that mainly present in the Indian saw scaled viper venom. The extract was tested with eight different concentrations i.e., 50µg to 400µg. The inhibitory activity was observed in dose dependent manner. The ethanolic extract of *Acalypha fruticosa* leaves was significantly ($p<0.005$) decreased the enzyme activity when the extract concentration was increased.

3.2.1 Inhibition of 5'Nucleotidase enzyme activity

The ethanolic extract of *Acalypha fruticosa* leaves was tested against the 5'Nucleotidase enzyme activity present in the saw scaled snake venom. The EEAF was showed a significant ($p<0.005$) inhibitory activity against the 5'Nucleotidase enzyme (figure 1a). The EEAF was treated with eight different concentrations (50µg - 400µg). Previously the venom was served alone in seven different concentrations (0.25ml to 1.75ml) to test the efficacy of the enzyme. Among them the 1.25ml concentration of venom was showed the maximum enzyme activity (figure 1). Hence the 1.25ml concentration was selected as optimum dose for inhibition studies.

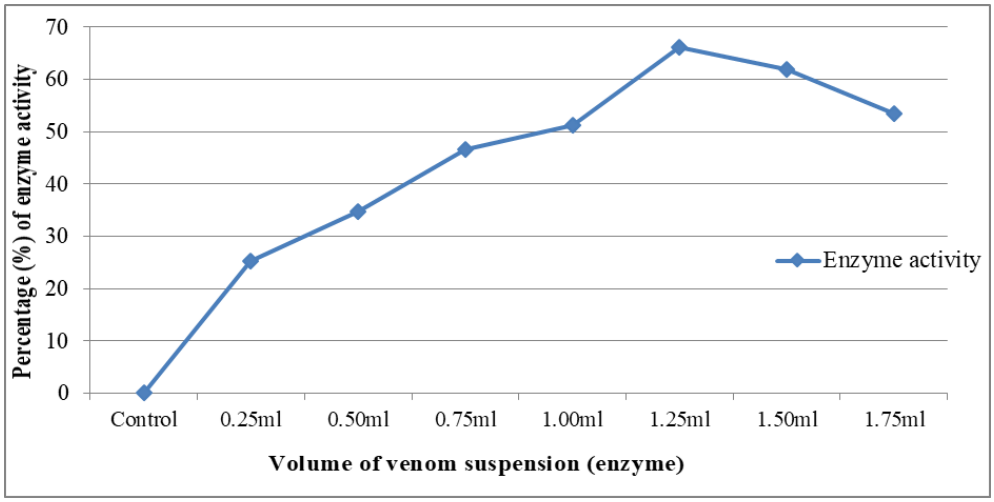

Fig 1: Efficacy of 5' Nucleotidase enzyme activity

The 1.25ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves for inhibition studies. In inhibition study, the EEAF was significantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibitory effect was observed at 250µg concentration of EEAF. This clearly indicates that the EEAF was significantly ($p<0.005$) inhibited the 5'Nucleotidase enzyme activity (figure 1a). The observed result was statistically significant ($p<0.005$) when compared to the control.

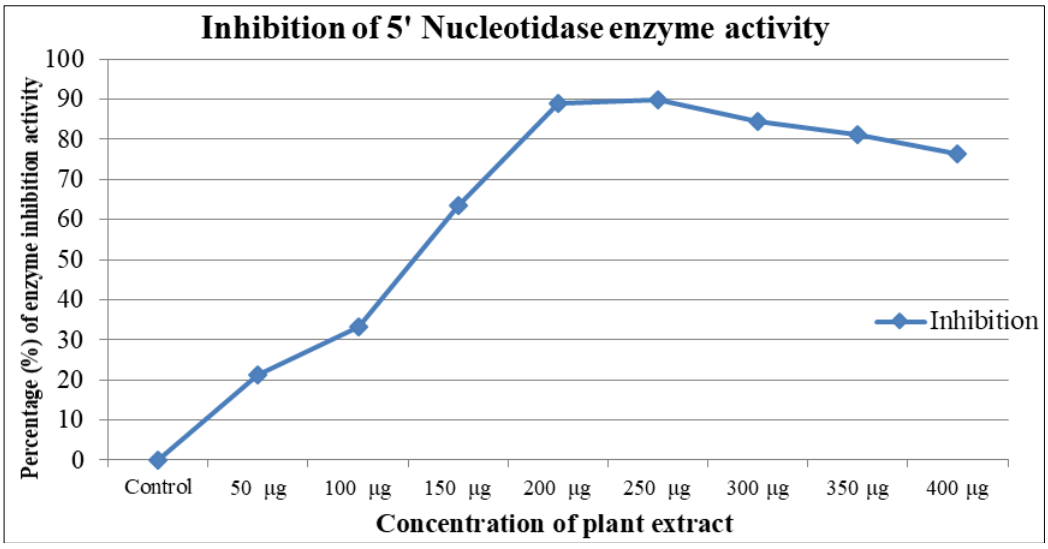

Fig 1a: Inhibition of 5' Nucleotidase enzyme activity by EEAF

3.2.2 Inhibition of phosphomonoesterase enzyme activity by EEAF

The ethanolic extract of *Acalypha fruticosa* leaves was tested against the phosphomonoesterase enzyme activity. The snake venom was served alone at eight different concentrations (0.1ml to 0.8ml) to test the efficacy of the enzyme. Among the 0.5ml concentration of snake venom possesses the maximum enzyme activity when compared to the other concentrations (figure 2). Hence, the 0.5ml concentration of venom was selected as optimum dose for the enzyme inhibition study.

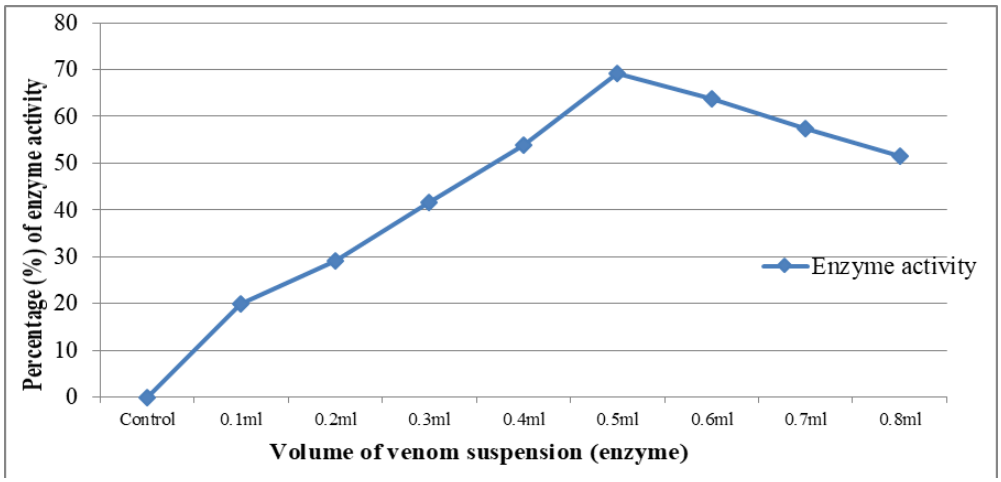

Fig 2: Efficacy of Phosphomonoesterase enzyme activity

In inhibition study, the 0.5ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves. In inhibition study, the EEAF was significantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibitory effect was observed at 250µg concentration of ethanolic extract of *Acalypha fruticosa* leaves. This clearly indicates that the EEAF was significantly ($p<0.005$) inhibited the phosphomonoesterase enzyme activity (figure 2a). The observed result was statistically significant ($p<0.005$) when compared to the control.

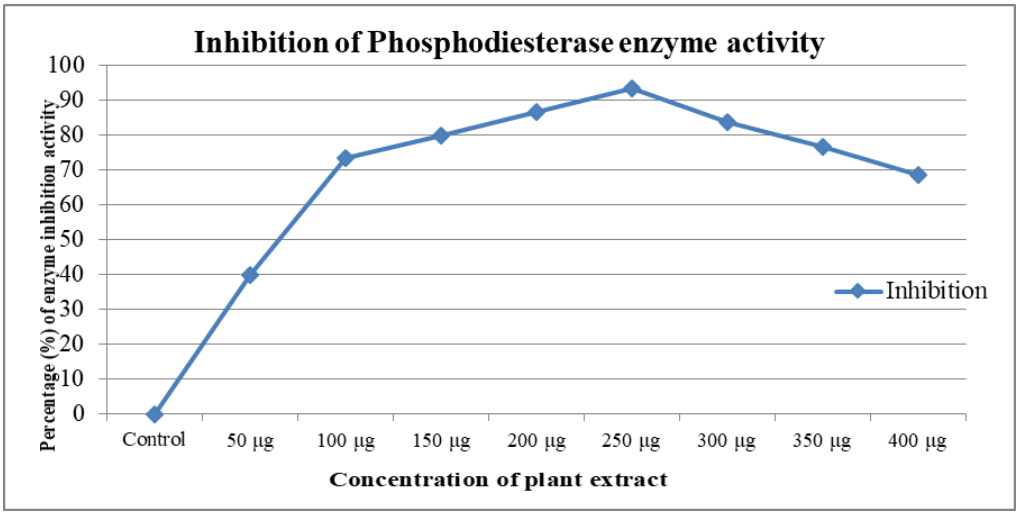

Fig 2a: Inhibition of Phosphomonoesterase enzyme activity by EEAF

3.2.3 Inhibition of Phosphodiesterase enzyme activity

The ethanolic extract of *Acalypha fruticosa* leaves was tested against the phosphodiesterase enzyme activity. Previously the venom of saw scaled viper was served alone at eight different concentrations (0.1ml to 0.8ml) to test the effectiveness of the enzyme. Among the tested concentrations the 0.5ml concentration of venom showed a maximum enzyme activity (figure 3). For this reason, the 0.5ml concentration of snake venom was selected as optimum dose for the inhibition study.

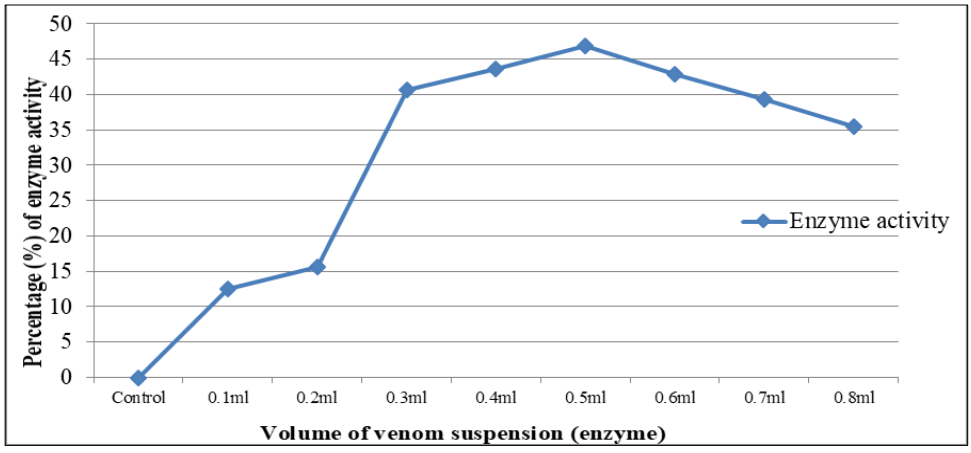

Fig 3: Efficacy of Phosphodiesterase enzyme activity

In inhibition study, the 0.5ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves. In inhibition study, the EEAF was significantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibition activity was observed at 250µg concentration of ethanolic extract of *Acalypha fruticosa* leaves. This result was clearly indicates that the EEAF was significantly ($p<0.005$) inhibited the phosphodiesterase enzyme activity (figure 3a). The observed result was statistically significant ($p<0.005$) when compared to the control.

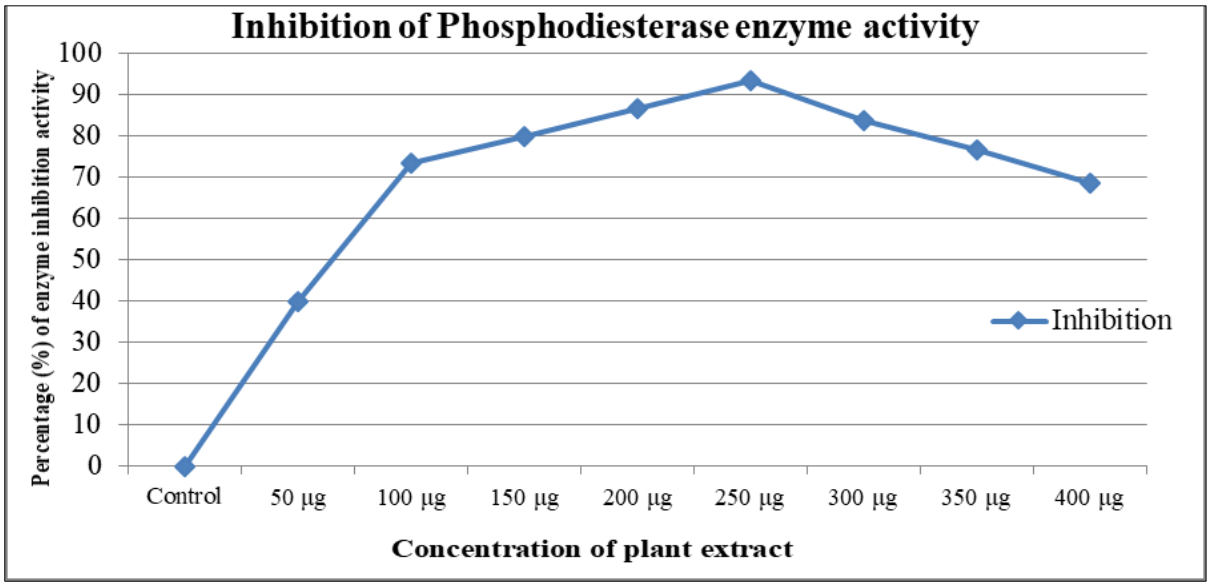

Fig 3a: Inhibition of Phosphodiesterase enzyme activity by EEAF

3.3.4 Inhibition of Acetylcholine esterase enzyme activity

The ethanolic extract of *Acalypha fruticosa* leaves was tested for the effectiveness against the acetylcholine esterase enzyme inhibitory activity. The venom was previously served alone at eight different concentrations (0.04ml to 0.40ml) to test the effectiveness of the Acetylcholine esterase enzyme present in the snake venom. Among all the tested concentrations, the 0.25ml concentration of snake venom showed a maximum enzyme activity (figure 4). Hence the 0.25ml concentration of snake venom was selected as optimum dose for the enzyme inhibition study.

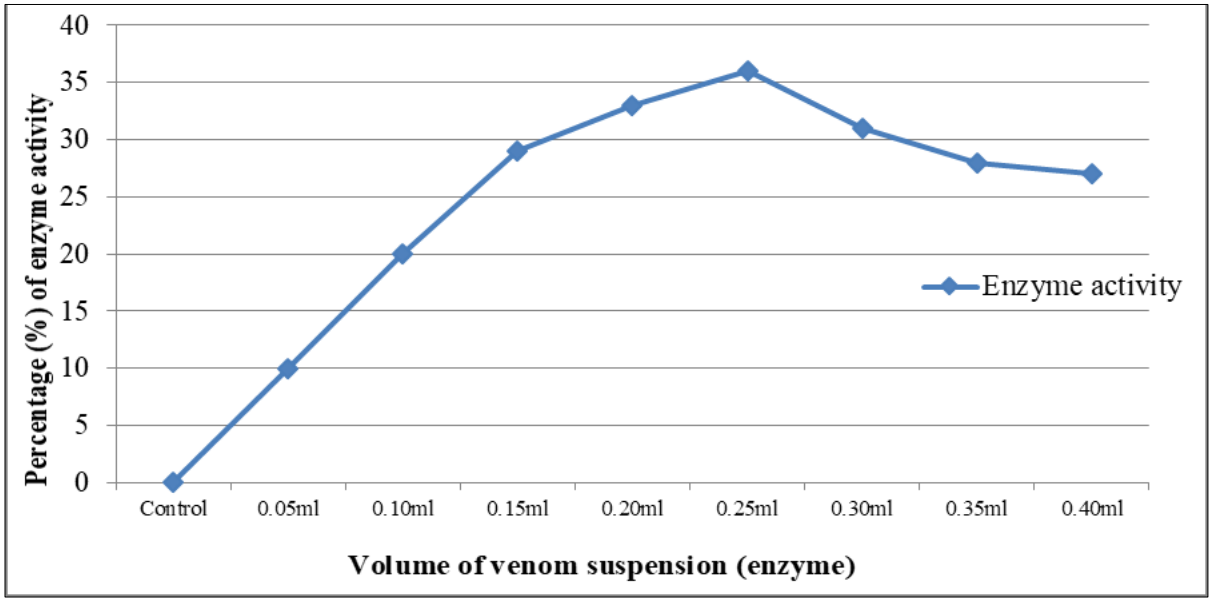

Fig 4: Efficacy of Acetylcholine esterase enzyme activity

In inhibition study, the 0.25ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves. In inhibition study, the EEAF was significantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibition activity was observed at 250µg concentration of ethanolic extract of *Acalypha fruticosa* leaves. This result was clearly demonstrates that the EEAF was significantly ($p<0.005$) inhibited the acetylcholine esterase enzyme activity (figure 4a). The observed result was statistically significant ($p<0.005$) when compared to the control.

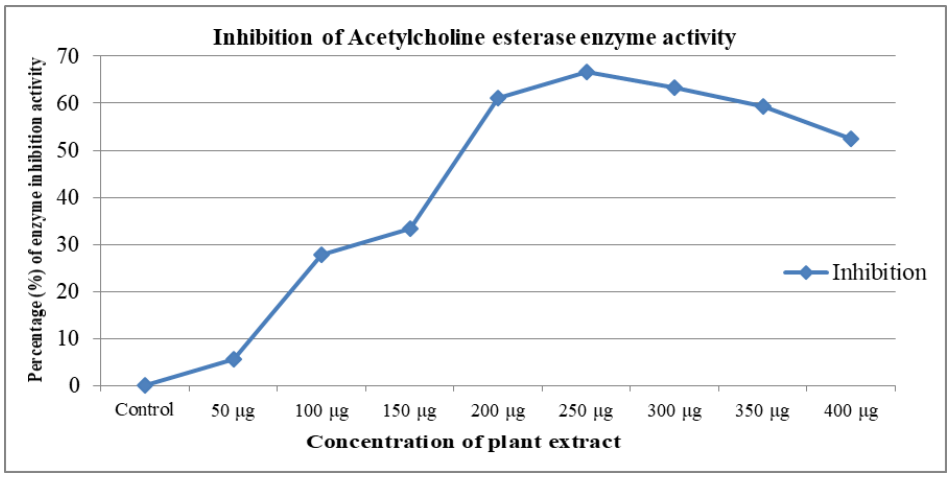

Fig 4a: Inhibition of Acetylcholine esterase enzyme activity by EEAF

3.3.5 Inhibition of Protease enzyme activity

The ethanolic extract of *Acalypha fruticosa* leaves was tested for the effectiveness against the protease enzyme inhibition activity. The venom was previously served alone at eight different concentrations (0.1ml to 0.8ml) to test the effectiveness of the protease enzyme present in the snake venom. Among all the tested concentrations, the 0.5ml concentration of snake venom showed a maximum enzyme activity (figure 5). Hence the 0.5ml concentration of snake venom was selected as optimum dose for the enzyme inhibition study.

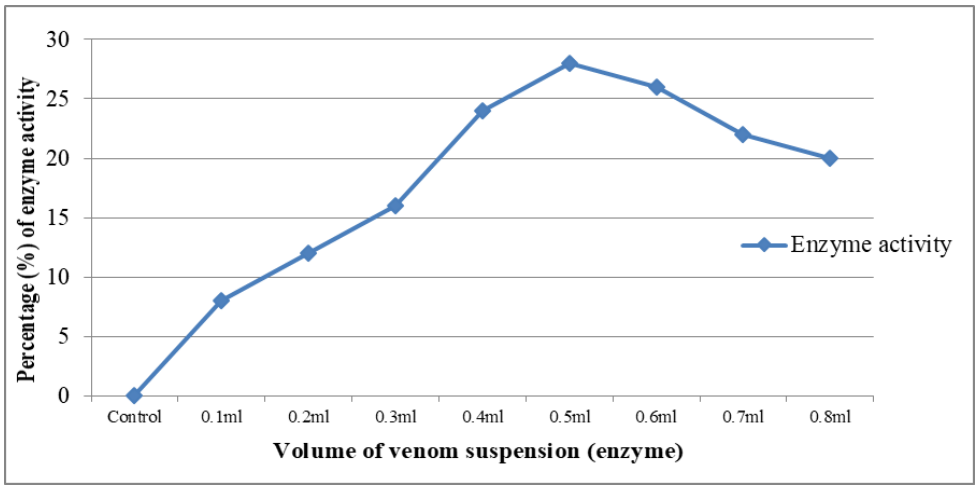

Fig 5: Efficacy of Protease enzyme activity

In inhibition study, the 0.5ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves. In inhibition study, the EEAF was significantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibition activity was observed at 250µg concentration of ethanolic extract of *Acalypha fruticosa* leaves. This result was clearly demonstrates that the EEAF was significantly ($p<0.005$) inhibited the acetylcholine esterase enzyme activity (figure 6a). The observed result was statistically significant ($p<0.005$) to the control.

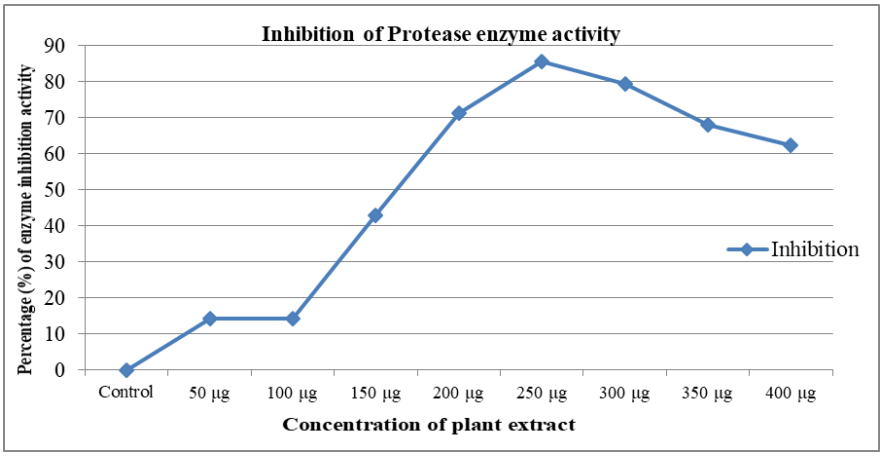

Fig 5a: Inhibition of Protease enzyme activity by EEAF

3.3.6 Inhibition of Hyaluronidase enzyme activity

The ethanolic extract of *Acalypha fruticosa* leaves was tested for the effectiveness against the hyaluronidase enzyme inhibition activity. The venom was previously served alone at eight different concentrations (0.01ml to 0.08ml) to test the effectiveness of the hyaluronidase enzyme present in the snake venom. Among all the tested concentrations, the 0.05mlconcentration of snake venom showed a maximum enzyme activity (figure 6). Hence the 0.05ml concentration of snake venom was selected as optimum dose for the enzyme inhibition study.

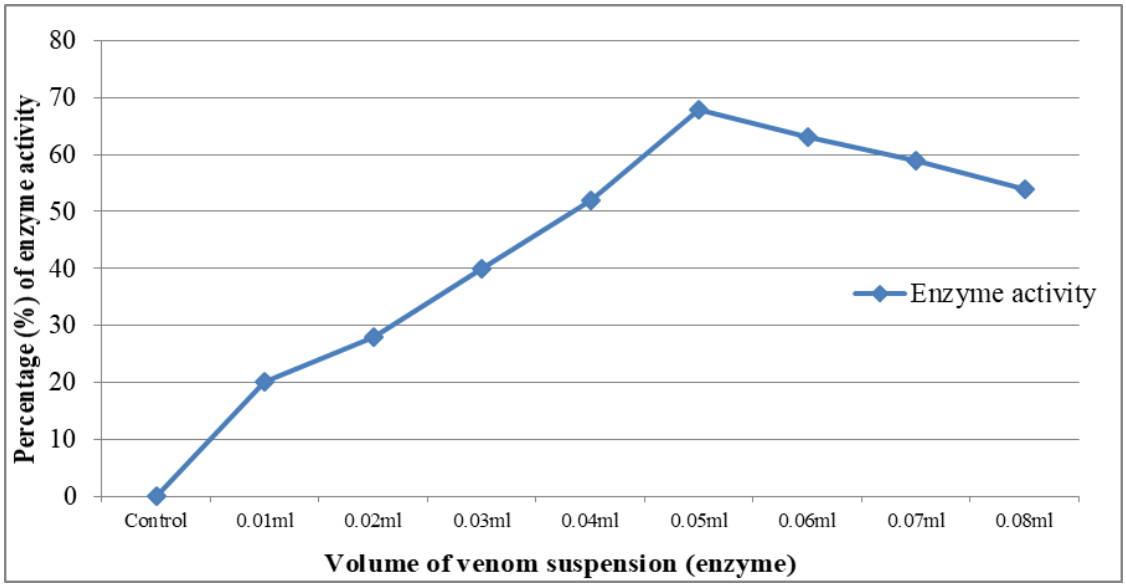

Fig 6: Efficacy of Hyaluronidase enzyme activity

In inhibition study, the 0.5ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves. In inhibition study, the EEAF wassignificantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibition activity was observed at 250µg concentration of ethanolic extract of *Acalypha fruticosa* leaves. This result was clearly demonstrates that the EEAF was significantly ($p<0.005$) inhibited the hyaluronidase enzyme activity (figure 6a). The observed result was statistically significant ($p<0.005$) to the control.

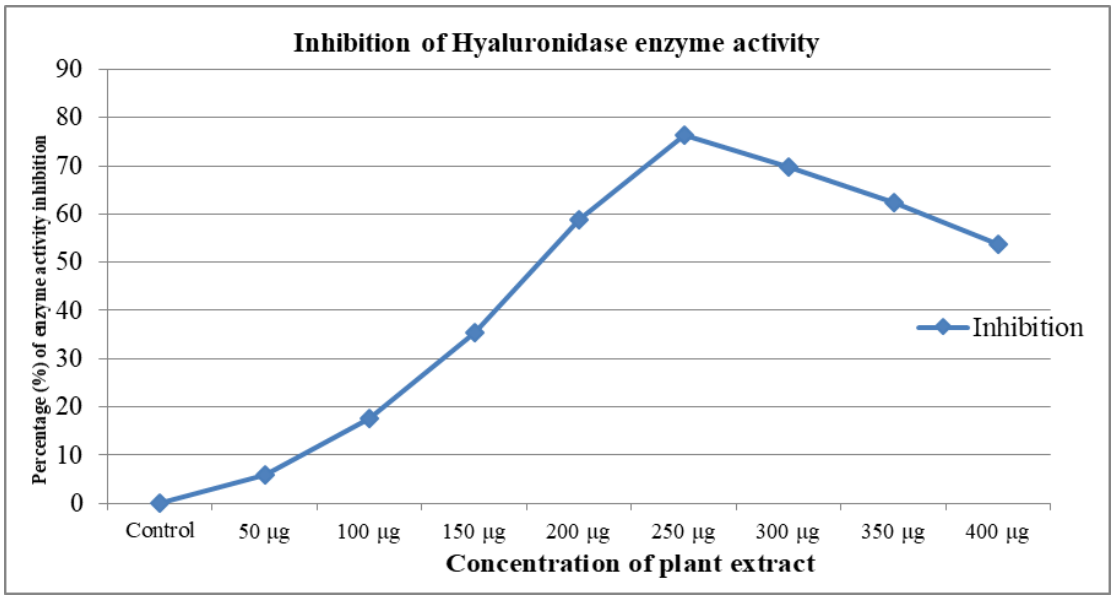

Fig 6a: Inhibition of Hyaluronidase enzyme activity by EEAF

3.3.7 Inhibition of Phospholipase A_2 enzyme activity

The ethanolic extract of *Acalypha fruticosa* leaves was tested for the effectiveness against the phospholipase A_2 enzyme inhibition activity. The venom was previously served alone at eight different concentrations (0.1ml to 0.8ml) to test the effectiveness of the phospholipase A_2 enzyme present in the snake venom. Among all the tested concentrations, the 0.5ml concentration of snake venom showed a maximum enzyme activity (figure 7). Hence the 0.5ml concentration of snake venom was selected as optimum dose for the enzyme inhibition study.

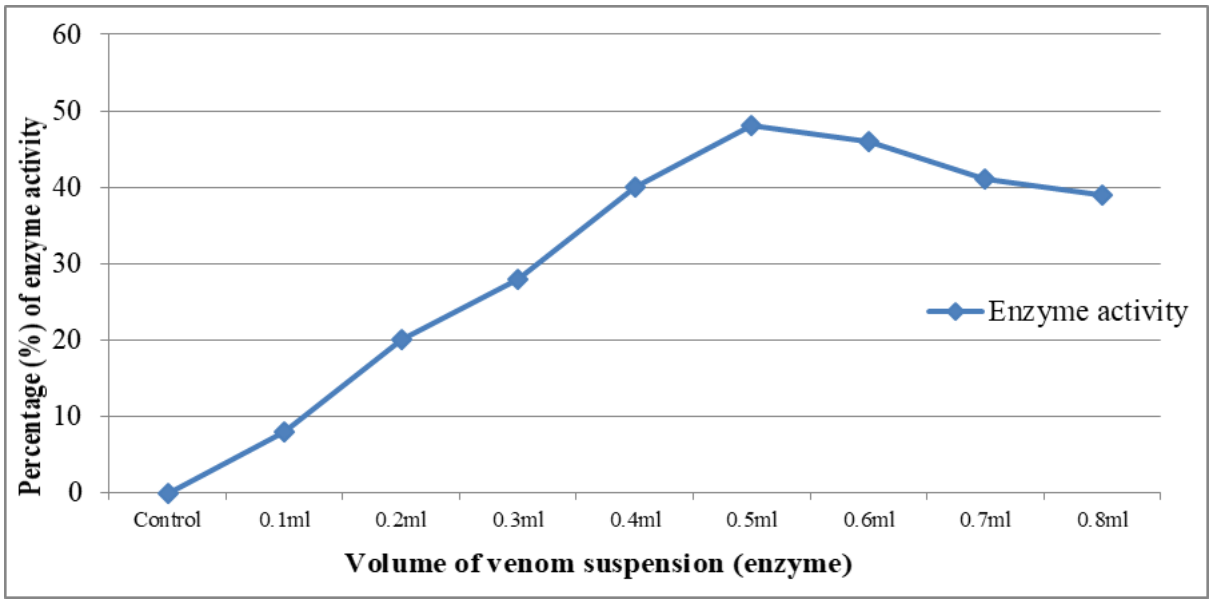

Fig 7: Efficacy of Phospholipase A_2 enzyme activity

In inhibition study, the 0.5ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves. In inhibition study, the EEAF was significantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibition activity was observed at 250µg concentration of ethanolic extract of *Acalypha fruticosa* leaves. This result was clearly demonstrates that the EEAF was significantly ($p<0.005$) inhibited the phospholipase A_2 enzyme activity (figure 7a). The observed result was statistically significant ($p<0.005$) to the control.

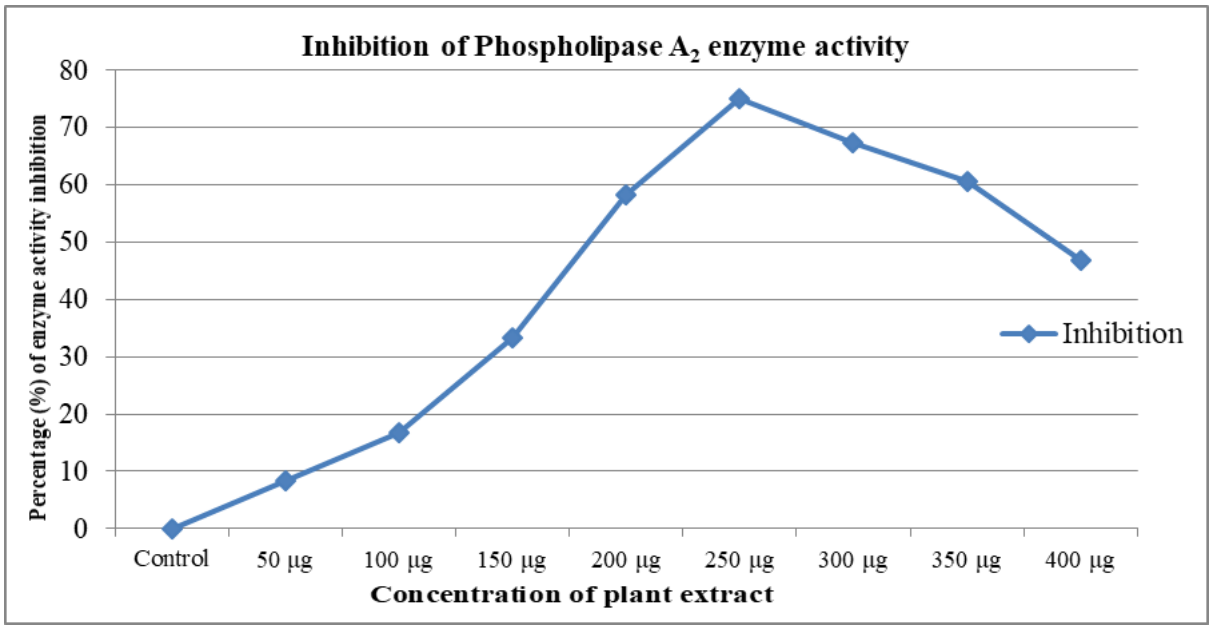

Fig 7a: Inhibition of Phospholipase A_2 enzyme activity by EEAF

3.3.8 Inhibition of L-Amino acid oxidase enzyme activity

The ethanolic extract of *Acalypha fruticosa* leaves was tested for the effectiveness against the L-Amino acid oxidase enzyme inhibition activity. The venom was previously served alone at eight different concentrations (0.04ml to 0.30ml) to test the effectiveness of the L-Amino acid oxidase enzyme present in the snake venom. Among all the tested concentrations, the 0.20ml concentration of snake venom showed a maximum enzyme activity (figure 8).Hence the 0.20ml concentration of snake venom was selected as optimum dose for the enzyme inhibition study.

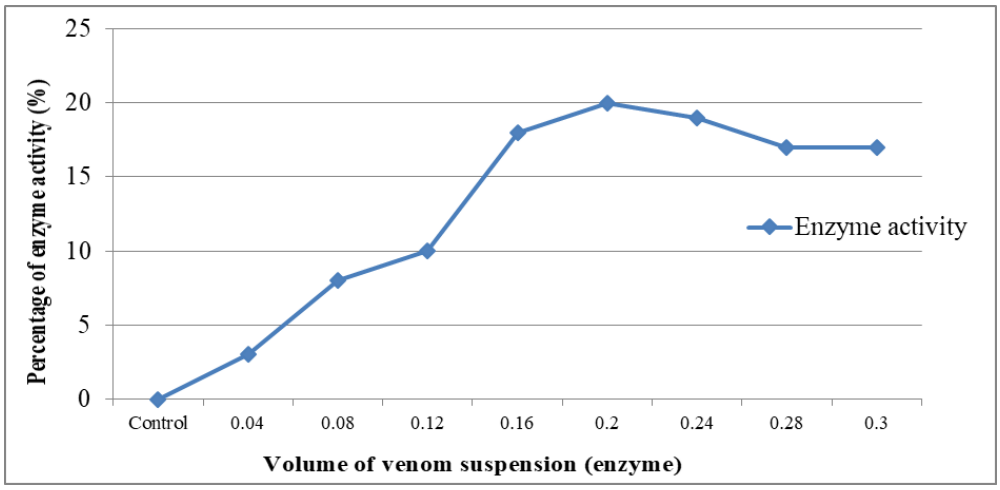

Fig 8: Efficacy of L-amino acid oxidase enzyme activity

In inhibition study, the 0.20ml of snake venom was preincubated for 30 minutes at 37˚C with eight different concentrations (50µg to 400µg) of ethanolic extract of *Acalypha fruticosa* leaves. In inhibition study, the EEAF was significantly ($p<0.005$) decreased the enzyme activity by dose dependant manner up to 250µg concentration when compared to the venom served alone. The maximum enzyme inhibition activity was observed at 250µg concentration of ethanolic extract of *Acalypha fruticosa* leaves. This result was clearly demonstrates that the EEAF was significantly ($p<0.005$) inhibited the L-Amino acid oxidase enzyme activity (figure 8a). The observed result was statistically significant ($p<0.005$) to the control.

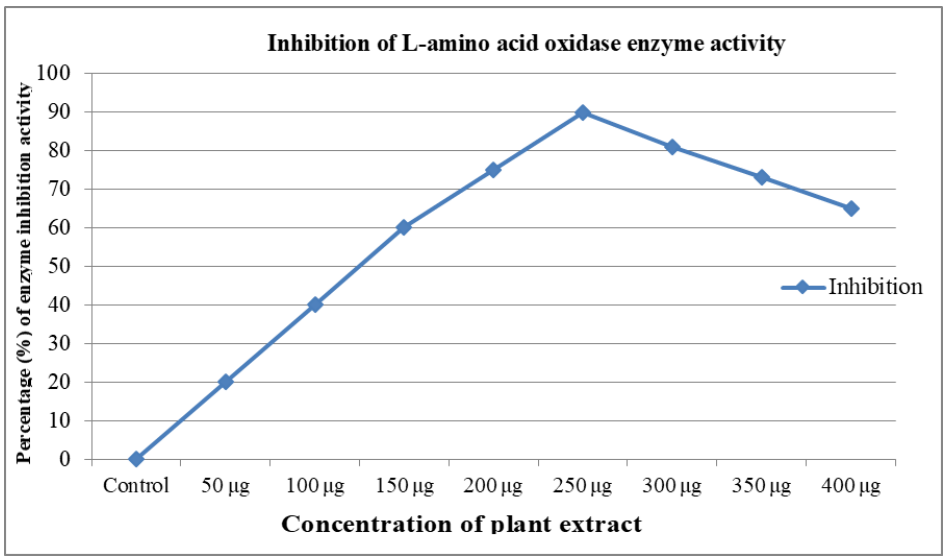

Fig 8a: Inhibition of L-amino acid oxidase enzyme activity by EEAF

4. Discussion

In the present study the qualitative phytochemical screening results was showed the presence of different level of phytochemicals such as flavonoids, carbohydrates, cardiac glycosides, phlobatannins, proteins, xanthoproteins, terpenoids, fatty acids and emodins in the different solvent extracts of *Acalypha fruticosa* leaves (Table 1). All of these compounds listed above are already well known for their biological activity. Microbial and plant products occupy the major part of the biologically active compounds discovered until now (Berdy *et al.*, 2005) [3]. The diethyl ether extract revealed the presence of flavonoids, coumarins, proteins terpenoids and xanthoproteins. The ethanolic extract revealed the presence of alkaloids, flavonoids, carbohydrates, glycosides, hydroxyl anthraquinones, proteins and phenols in moderately high concentration (Table 1). Flavonoids belong to the group of polyphenolic compounds and are typically known for health promoting properties such as antioxidant, antiallergic, anti-inflammatory, antimicrobial and anticancer properties (Aiyelaagbe and Osamudiamen, 2009) [1]. They exist widely in the plant kingdom and displayed positive correlation between increased consumption of flavonoids and reduced risk of cardiovascular and cancer diseases (Yang *et al.*, 2001) [30].

The ethyl acetate extract showed the presence of alkaloids and glycosides in high and moderately high concentration respectively and carbohydrates, proteins, xanthoproteins, terpenoids, phenols, resins and fatty acids in small concentration. Alkaloids have been reported to possess analgesic, antispasmodic and bactericidal, antimalarial and analgesic activities (Okwu and Okwu, 2004; Oomah, 2003) [20, 21]. The methanol extract showed the presence of glycosides in high concentration and alkaloids, flavonoids, carhohydrates, saponins, hydroxyl anthraquinones, terpenoids and phenols in small concentration (Table 1). Terpenoids such as triterpenes, sesquiterpenes and diterpenes have been referred to as antibiotics, insecticidal, anthelmintic and antiseptic in pharmaceutical industry (Parveen *et al.*, 2010) [22].

The antivenom currently used for ophidian bites is a suspension of antibodies, prepared mainly from horses. Animals are hyper immunized against the venom of a given specific species (monovalent) or venom from several different species at the same time (polyvalent). Infusion of antisnake venom may lead to adverse reactions ranging from early reactions (pruritus, urticaria) to potentially fatal anaphylaxis. The reported incidence of these reactions varies from 5 to 80%. There are also pyrogen reactions due to endotoxin contamination. Serum sickness may also develop in certain cases (Dhanya *et al.,* 2009) [7].

The plant kingdom provides a perfect alternative to antisnake venom. Medicinal plants have been used as folk medicine for treatment of snake bites in many populations. Reliance on medicinal plants is primarily due to their safety, effectiveness, cultural preferences, inexpensive nature and dependence on neighboring forests. Globally, traditional healers are practicing herbal medicine to cure snake envenomations. However, the practice is not really recognized by modern medicine. The number of studies has evaluating the pharmacologically active principles against snake bites are few. Though novel phytotherapeutic agents have been isolated from plants due to vital leads from ethnic groups, yet validation is still an issue. Emphasis should be on proper design of both *in vivo* and *in vitro* studies, so that they relate exactly to the clinical situations. The indigenous systems of medicine use medicinal plants for the treatment of snake bites. There is a huge repository of plants reported to possess antisnake venom activity (Coe and Anderson, 2005; Tsai *et al.,* 2007) [6, 28]. Enzyme inhibiting properties have been associated with chemically active compounds of flavonoids, polyphenols, terpenoids, xanthene etc. The phytochemicals also inhibit PLA_2 activities of viper and cobra venom. Phenolics, especially polyphenols, like some tannins bind proteins, acting upon components of venom directly and disabling them to act on receptors. They could also act by competitive blocking of the receptors. Tannic acid has been found to be a potent inhibitor of hyaluronidase (Januario *et al.,* 2004; Chatterjee *et al.,* 2004) [15, 5].

In the present study, the ethanolic extract of *Acalypha fruticosa* leaves was tested against the eight different enzymes that present in Indian saw scaled viper venom which is responsible for the lethality. The ethanolic extract showed a potent enzyme inhibitory effects, the EEAF was showed a significant ($p<0.005$) snake venom inhibition activity. It may due to the presence of various phytochemicals present in the *Acalypha fruticosa* leaves. The efficacy of the snake venom enzymes was previously assayed by administering them in alone. The observed results showed the decreased levels of substrate. When the venom concentration was increased the substrate level was significantly ($p<0.005$) decreased. It indicates the activeness of the enzymes present in Indian saw scaled viper venom. Then the venom was tested with 5 different concentrations i.e., 50µg to 250µg of EEAF. The snake venom was preincubated with the different concentrations of plant extract. The enzyme inhibitory activity was observed in dose dependant manner. When the extract concentration was increased the activity of the enzyme was significantly ($p<0.005$) decreased. The 250µg concentration of ethanolic extract of showed the maximum inhibitory activity in all tested enzymes (Figure 1-8).

Screening of plants used in traditional medicine and determination of their active principles and different activities is being undertaken. The active principles isolated have been associated with various pharmacological properties and may provide a substantial contribution to the modern therapeutics of snake bite. A thorough literature survey highlights the fact that plant kingdom has tremendous resources which can be exploited for unidentified novel compounds with antivenin activity or those supplementing the action of antisnake venom. In the present study an attempt has been made to present a comprehensive account of antisnake venom activity of *Acalypha fruticosa* leaves for the treatment of snake bite. The results clearly showed that the plant *Acalypha fruticosa* leaves was effective antisnake venom activity against Indian saw scaled viper venom.

5. Conclusion

Because of various limitations of antisnake venom serum, herbal therapeutics for snake envenomations seems to be a perfect alternative. In view of a plethora of active compounds in the *Acalypha fruticosa* leaves, an in depth scientific investigation is warranted to evaluate their antisnake venom potential, to derive therapeutically effective natural products for snake bites. Complete phytochemical investigation of extracts and analysis of active principles to be used as potent therapeutic agents along with well-designed studies evaluating the pharmacologically active principles are necessary. Further, standardization of the basic active compound along with toxicity and safety studies is mandatory.

6. References

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