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In vitro antivenom and antioxidant potential of *Vitex negundo* leaves (green and blue) against Russell's viper (*Daboia russelli*) and Indian cobra (*Naja naja)* venom

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ABSTRACT

In the present investigation hydroethanolic extracts of Vitex negundo leaves (Blue and green colour) were evaluated for their antioxidant, antiplatelet, antihemolytic and in-vitro antivenom potential against Naja naja and Daboia russelii venoms. Preliminary phytochemicals followed by GCMS analysis was carried out to identify possible chemical compounds. Both the extracts exhibited free radical scavenging effects against DPPH, OH^- , NO^- radicals and H_2O_2 in concentration dependant manner. However, blue leaf of V.negundo exhibited better scavenging effect when compared with green leaf. Various pharmacological potentials such as antihemolytic, antiproteolytic, antifibrinolytic and phospholipaseA_2 inhibition were assessed and effective venom neutralizing potential was proven upon in-vitro treatment with the extracts of Vitex negundo leaves. Findings of the present study revealed that blue leaf extract of Vitex negundo has exerted potent antioxidant and venom neutralizing effect when compared with green leaf extract. This could be due to the presence of various phytoconstituents in both blue and green leaf extract which would help to develop potent antidote therapy against Naja naja and Daboia russelii envenomation.

Keywords: *Naja naja*, *Daboia russelii* , Antiplatelet , Antioxidant , Hemolysis, *Vitex negundo*

INTRODUCTION

Snake bite is an important public health issue in rural regions of tropical and subtropical countries located in Asian, Africa and Laten America [1-2]. More than 2000 species of snake in this world and 216 species in India have been identified among which 52 are highly venomous. Cobra (*Naja naja*), Krait (*Bangarus caeruleus*), Russell's viper (*Daboia russelli*) and Saw scaled viper (*Echis carinatus*) are commonly considered be poisonous snakes in the Indian ethnicity [3]. Since development of snake venom antiserum and its standardization are found to be expensive and difficult, the efforts are continuously being made to invent alternative treatment strategy from medicinal plants [4].

Envenomation of snake bite is caused due to the nature of enzymatic and non-enzymatic toxic compounds present in the poison glands [5]. Phospholipase A_2 (PLA_2), neurotoxins and cardiotoxins are the major classes of cobra venom polypeptides involved in the toxicity and pharmacology of snake bite. Alkaloids, acids, coumestin, steroids, glycoproteins, glycosides, phenols, pterocarpenes, tannins, terpenoids, quinonoid xanthine and miscellaneous chemical groups present in the plant extracts possess effective antidote against snake venom envenomation [6].

Though the definite mechanism of herbal based antidote therapy is not yet delineated, majority of the herbal compounds tend to neutralize the toxic venom constituents [7]. Neutralization of venom by phytoconstituents includes the process of enzyme inactivation, chelation, adjuvant actions, anti-oxidation and protein folding [8-9].

Vitex negundo (Verbenaceae) known as the five-leaved chaste tree is a large aromatic shrub with quadrangular, densely whitish to mentose branchlets. South and Southeast Asians widely use this herb in folk medicine [10]. *Vitex negundo* is native to tropical eastern and southern Africa and Asia. There are two types of leaves seen with respect to their color: green and blue leaves. The leaves are traditionally documented to possess potent pharmacological properties like anti-inflammatory, anti-rheumatic, antibiotic, hepatoprotective, antioxidant, anti-convulsant, anti-androgen, snake venom neutralization and anti-allergic activities [11]. It is in this context that the present study was undertaken to explore the antivenom potential of the blue and green leaves of *Vitex negundo* and compare their venom neutralization potential, respectively.

MATERIALS AND METHODS

2.1. Plant Material Collection and Extraction

The plant samples of *Vitex negundo* (blue and green leaves) were collected in and around Erode and Coimbatore, respectively. The leaves were then shade dried for a week and powdered by manual grinding. 25g of the powder was then dissolved in 250ml of 50% ethanol (hydroethanol) and kept for 48-72hrs. The filtrate was then lyophilized and the lyophilized sample was then used for further *in-vitro* studies. Phytochemicals were qualitatively analyzed in the leaf extracts as described by Khandelwal [12].

2.2. GC-MS Analysis

Gas Chromatography Mass Spectroscopy (GC-MS) is usually performed to identify and isolate active constituents from a plant extract. The lyophilized samples were dissolved in GC grade methanol; the samples were filtered using Syringe filter prior to the GC-MS injection. 1µl of the samples were taken for injection and standard operating procedure for SHIMADZU GCMS was undertaken.

2.3. Radical scavenging activity assays

The determination of free radical scavenging activity of *Vitex negundo* fruit extract against DPPH, NO, OH^- and $H_2O_2$was undertaken by described standard methods [13-16]. Total antioxidant capacity was determined on the basis of ability of antioxidant to form colored complex with potassium Ferricyanide, TCA and ferric chloride [17].

2.4. Antiplatelet assay

Platelet rich plasma 0.13 x 10^{-7} for each assay was re-suspended in tyrode buffer (pH adjusted to 7.4 with 0.25M HCl). Aggregation of platelets was induced by $CaCl_2$ at a final concentration of 2µM.Platelet aggregation was recorded by increasing transmittance value of spectrophotometric measurements. Different concentrations (100,200,300,400 and 500µg) of plant extract were added to the platelet suspension for 1minute, exposed at 37^{o}C before treatment with platelet aggregating agents. Aspirin at 500µg/ml was used as standard to determine the *in-vitro* antiplatelet aggregation property [18].

2.5. Antihemolytic assay

Blood was collected from healthy adult human volunteers and collected in sterile Alseiver's solutions and used within 5hours of collection. The preparation of cell suspension was carried out by previously described method [19]. In a series of test tubes, take 800µl of 1% w/v Triton-X-100 and make it up to a volume of 3ml with phosphate buffer. Similarly, take 3ml of distilled water. This serves as a positive control. In another series of tubes, add different concentrations of the plant extract (100-500µg) which was previously incubated with both cobra and russell's viper venom for 30minutes (venom was dissolved in saline and used). To all the tubes, add 500µl of RBC suspension or 100µl from packed cell volume and mix gently. Incubate the tubes in a water bath at 37^{o}C for 1hour. Centrifuge the contents at maximum speed for 5minutes. Collect the supernatant and take the absorbance at 541nm against phosphate buffer as blank. From the reading, calculate the percentage inhibition of hemolytic activity.

2.6. Anti-venom activity and venom neutralization

2.6.1. Proteolytic activity

The process of inhibition of casein digestion induced by cobra and russell's viper venom by blue and green leaves of *Vitex negundo* was used as a measure of its proteolytic inhibition activity [20]. A solution of 1% (w/v) casein in 20

mM phosphate buffer (containing 150 mM NaCl, pH 8) was incubated with different concentrations (protein mg/ml) of the plant extract (100-500µg) along with cobra and russell's viper venom for 1hr at 37°C. Thereafter, 0.5 ml of 10% TCA was added and the protein concentration in the supernatant was determined at 625nm. Proteolytic activity was calculated using trypsin as a standard protease.

2.6.2. Procoagulant activity

Procoagulant activity was assayed according to the method described previously as modified by another study [21]. Various amounts of venom dissolved in normal saline were added to human citrated plasma at 37^{o}C. Coagulation time was recorded and the minimum coagulant dose (MCD) was determined as the venom dose which induced clotting of plasma within 60seconds. In neutralization assays, constant amount of cobra and russell's viper venom was mixed with various dilutions of plant extracts (1.2, 1.4, 1.6, 1.8, and 2.0mg/ml). The mixtures were incubated for 30minutes at 37^{o}C. Then, 0.1ml of mixture was added to 0.3ml of citrated plasma and the clotting time recorded. In control tubes, plasma was incubated with either venom or plant extracts alone. Neutralization was defined as effective dose which was able to completely neutralize coagulant activity.

2.6.3. Phospholipase A2 activity

Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose-egg yolk gel plate by the methods described in other studies [22]. Increasing doses of cobra and russell's viper venom were added to 3 mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10mM $CaCl_2$. Plates were incubated at 37^{o}C overnight and the diameters of the hemolytic halos were measured. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11mm diameter. The efficacy of plant extracts in neutralizing the phospholipase activity was determined by mixing constant amount of venom with various amount of plant extracts (0.6, 0.8, 1.0, 1.2 and 1.4mg/ml) and incubated at 37^{o} C for 30 minutes. Then aliquots of 10µl of mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Plates were incubated at 37^{o}C for 20 hours. Neutralization was expressed as concentration of plant extract which could reduce the hemolytic halo by 50% when compared to the effect induced by venom alone.

2.6.4. Fibrinolytic activity

A modified plaque assay was used. The minimum fibrinolytic dose was defined as amount of venom that caused a fibrinolytic halo of 10mm diameter. Neutralization experiments were performed by incubating a constant amount of venom with various concentrations of plant extracts (0.6, 0.8, 1.0, 1.2, and 1.4mg/ml) at 37^{o}C for 1hour. After incubation, the mixture was applied to the wells in the plates. After 18hours of incubation at 37^{o}C, fibrinolytic halos were measured [23-24].

2.7. Statistical Analysis

The results obtained were expressed as the mean $\pm$ SD of triplicates of experiments done, with $p<0.05$.

RESULTS

3.1. Qualitative phytochemical screening

The preliminary phytochemical screening has shown that the presence of alkaloids, flavonoids, saponins, carbohydrates, proteins, phenols, tannins, steroids and glycosides in the leaf extracts. From the table, it was evident that all solvent extracts were found to contain selected phytochemicals. However, when compared with different solvent extracts, hydroethanolic extract was found to contain most of the selected active phytoconstituents screened for both blue and green leaves of *Vitex negundo*. The summary of the results is presented in the table 1.

3.2. GC-MS analysis

Samples were subjected to GC-MS run for 40 minutes using DB-5 column. 14 compounds in blue leaf and 11 in green leaf were found to contain in hydroethanolic extract of *V.negundo*. The predominant peak for blue leaves was obtained at the retention time of 4.843 with a peak area of 484110 which corresponds to beta phellandrene, a major cyclic monoterpene. 13 other minor compounds were also found to be present in the extract. In the green leaf extract, the predominant peak was obtained at a retention time of 23.006 with a peak area of 549265, which corresponds to taucadinol. 10 other minor compounds were also present along with taucadinol in the leaf extract. The chromatograms showing the predominant compounds in blue and green leaves have been pictorially represented in Figure (1 & 2).

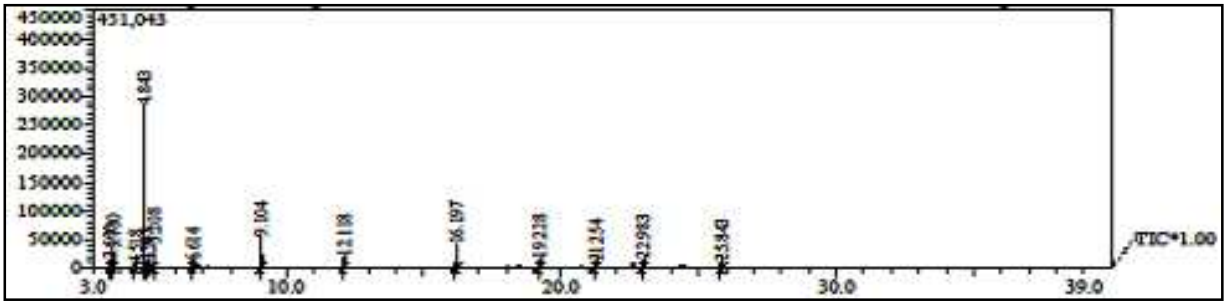

Figure 1: GC-MS chromatogram of chemical constituents in hydroethanolic extract of *Vitex negundo* green leaf

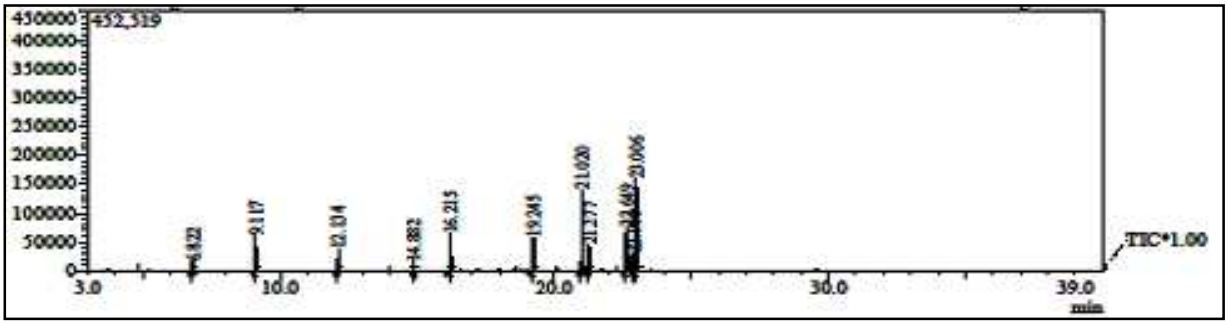

Figure 2: GC-MS chromatogram of chemical constituents in hydroethanolic extract of *Vitex negundo* blue leaf

Table 1: Preliminary Phytochemical screening of Green and Blue leaves of *Vitex negundo* in different solvent extracts

Phytochemicals	Green Leaves			Blue Leaves		
	50% Ethanol	Ethyl acetate	Aqueous	50% Ethanol	Ethyl acetate	Aqueous
Alkaloids						
•Dragendroff test	++	-	+	++	-	-
•Wagner test	+	-	-	+	-	-
•Meyer test	-	-	-	+	+	-
Flavonoids	+	+	+	++	-	+
Saponins	+	-	-	++	-	+
Carbohydrates						
•Fehlings test	++	-	++	++	-	++
•Benedicts test	-	+	+	+	+	+
Proteins						
•Biuret test	+	-	+	++	-	+
•Millon's test	+	-	-	+	+	+
Phenols						
•$Fecl_3$	++	-	-	++	-	+
•Lead acetate	++	-	-	+	-	+
Tannins						
•$Fecl_3$	++	-	-	++	-	+
•Lead acetate	+	-	-	+	-	+
Steroids						
•Salkowsi reaction	-	-	-	-	-	+
Glycosides	-	+	-	-	+	-

+ Present, - Absent

3.3. In-vitro antioxidant activity

The reducing power activity, DPPH, NO, OH ions and H2O2 scavenging capacity of hydroethanolic extract of blue and green variety leaves were compared with standard ascorbic acid (Figure 3,4,5,6 and 7).

DPPH Radical Scavenging Activity

The blue leaf extract has shown better free radical scavenging effect of DPPH than green leaf extract in a concentration dependant manner; however they are less in comparison to the standard ascorbic acid. The IC_{50} values

were found to be 340.23, 360.15, and 242.5 µg for blue leaf, green leaf and ascorbic acid respectively. The comparison of IC_{50} values of the standard ascorbic acid and different leaf extract of *Vitex negundo* was graphically represented in Figure 3.

DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl to decolorize from purple to yellow in presence of antioxidants [25]. Our study demonstrated the ability of Blue and Green leaves of *Vitex negundo* to significantly scavenge DPPH free radical [26].

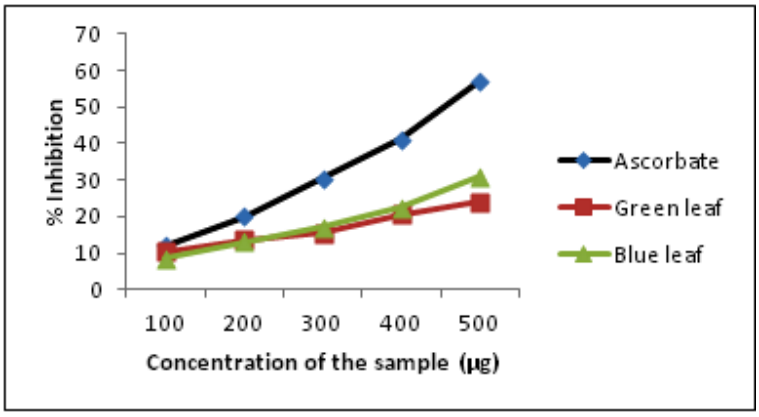

Figure 3: Radical scavenging activity of *V.negundo* leaf extracts and ascorbic acid against DPPH radical increase at increasing concentration. Values are the average of triplicates and expressed as mean ± standard deviation

NO Radical Scavenging Activity

50% and 45.5% Nitric oxide radical scavenging activity were exerted by hydroethanolic extract of blue and green leaves of *Vitex negundo* respectively (Figure 4). The percentage inhibition of NO radical by hydroethanolic extract of blue and green leaves of *Vitex negundo* were shown in Figure 4. NO radical scavenging effect of blue and green leaves was observed to be 50% and 45.5% at the 500µg/ml concentration respectively. The IC_{50} values of blue and green leaves of *V.nedungo* and ascorbate were found to be 297, 307 and 254.3 µg. Nitric oxide plays a mediator role in physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity [27]. In the present study, the nitrite produced by the incubation of sodium nitropruside solution in the standard phosphate buffer at 25^0 C was reduced by hydroethanolic extract of blue leaf. This might be due to the antioxidant principles in the extract which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite [28].

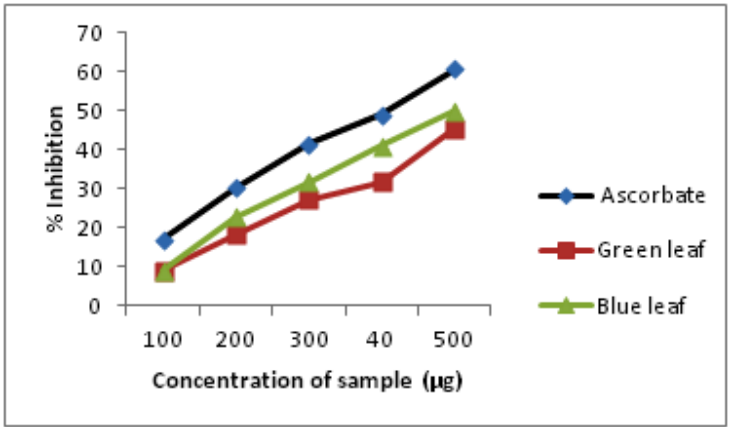

Figure 4: Concentration dependant radical scavenging activity of *V.negundo* extracts and ascorbic acid against NO radical. Values are the average of triplicates and expressed as mean ± standard deviation

Hydroxyl Radical Scavenging Activity

Both blue and green leaf extracts of *Vitex negundo* exhibited dose dependent scavenging activity against hydroxyl radical with maximum inhibition of 59.1% and 54.55% at 500µg (Figure 5). However, standard ascorbate was found to possess maximum inhibition. The protective action of blue and green leaves of *Vitex negundo* might be due to presence active phytochemicals which significantly inhibited the formation of hydroxyl radical. Similarly 70% aqueous acetone extract of *Machilus zuihoensis* and *Centaurium erythraea* effectively inhibited the formation of hydroxyl radical generated in Fenton reaction which supported our findings [29-30].

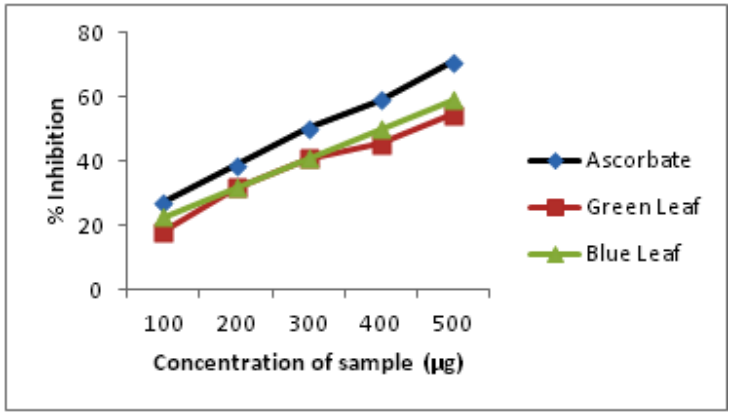

Figure 5: Hydroxyl radical scavenging activity of *V.negundo* extracts and ascorbic acid increase concentration dependant manner. Values are the average of triplicates and expressed as mean ± standard deviation

H_2O_2 Scavenging Activity

Hydrogen peroxide (H_2O_2) is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly, and once inside the cell, it can react with Fe^{2+} and Cu^{2+} ions to form hydroxyl radical and this might be the origin of many of its toxic effects [31]. Hydrogen peroxide scavenging activity of blue and green leaf extract was found to be 31.4% and 30.6% respectively, while that of standard ascorbate was 74.7% (Figure 6). From the results, it appeared that H_2O_2 scavenging activity of *V.negundo* is negligible when compared with standard ascorbic acid. In this assay, IC_{50} values of blue, green leaf extracts, and ascorbate were found to be 332, 340 and 267µg respectively. Earlier report revealed that methanolic extract of *Jasminum sambac* exhibited significant hydrogen peroxide scavenging activity which is similar to our investigation [32].

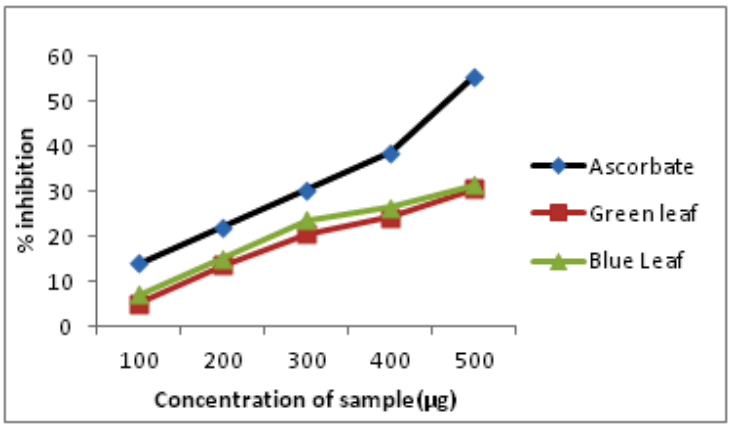

Figure 6: Radical scavenging activity of *V.negundo* leaf extracts and ascorbic acid against H_2O_2 increase at increasing concentration. Values are the average of triplicates and expressed as mean ± standard deviation

Reducing Potential

As illustrated in the Figure 7, the maximum reducing potential of blue and green leaves were found to be at 0.64 and 0.51 absorbance respectively which was observed at a maximum concentration of 500µg/ml. However, they were found to be significantly lower when compared with standard ascorbate (0.73 absorbance). The presence of reducing agents in blue and green leaves of *Vitex negundo* extract causes the reduction of the Fe^{3+}/ Ferricyanide complex to the ferrous form by donating hydrogen atom, which was performed to measure the reducing capacity. Shanmugam *et al* and Sharma *et al* have reported that *Canthium parviflorum* and *Ficus racemosa* root possess potent reducing power which supported our findings [33-34].

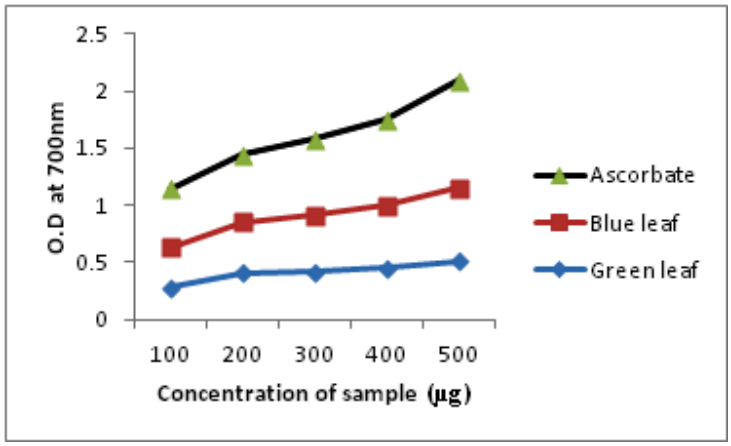

Figure 7: Reducing potential of test *V.Negundo* leaf extracts and standard ascorbic acid increased. Values are the average of triplicates and expressed as mean ± standard deviation

3.4. Antihemolytic and Antivenom Activity

The *in-vitro* antiplatelet study of hydroethanolic extract of blue and green leaves of *Vitex negundo* showed considerable antiplatelet activity at 500µg/ml concentration. The antiplatelet activity was found to be dose dependent but it was found to be considerably lower when compared to standard aspirin. Among the two leaf extracts, blue leaves inhibited more efficiency in inhibiting platelet aggregation than green leaf extract (Figure 8A and 8B). The anti-hemolytic potential was observed to be directly proportional to the concentration of sample extracts. The highest inhibition of 71.2% and 67.2% was found at 500µg/ml concentration for blue and green leaves respectively. Thus, the blue leaves were found to substantially inhibit hemolysis caused by triton-X-100 than the green leaves (8C).

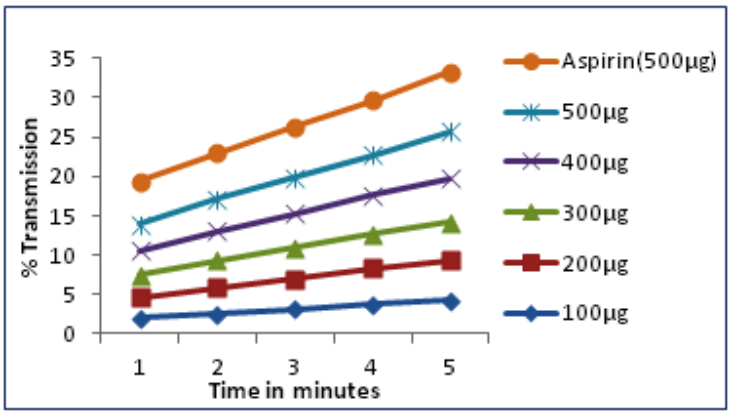

Figure 8 A: Anti-platelet Activity of Green Leaves of *Vitex negundo* in different time intervals at increasing concentration manner. Values are the average of triplicates and expressed as mean ± standard deviation

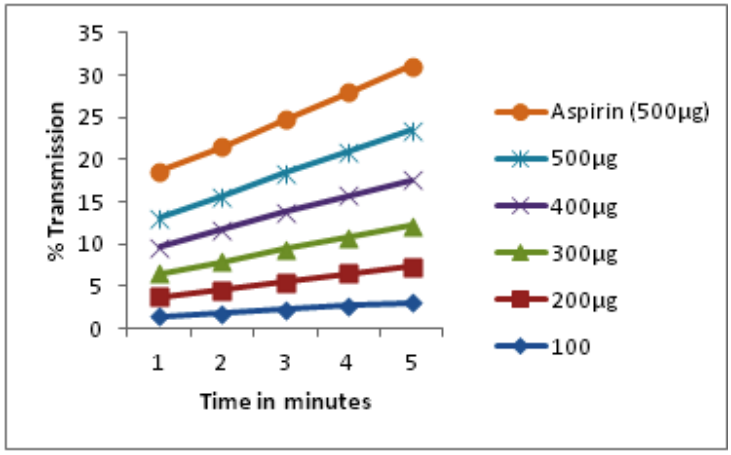

Figure 8 B: Antiplatelet Activity of Blue Leaves of *Vitex negundo* in different time intervals at increasing concentration manner. Values are the average of triplicates and expressed as mean ± standard deviation

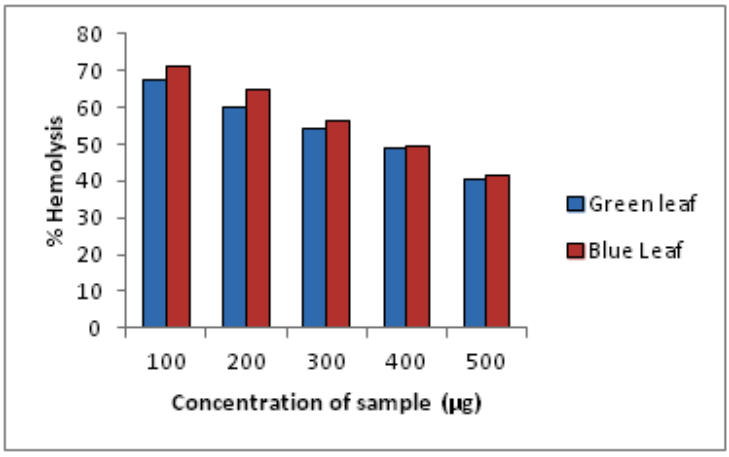

Figure 8C: *V.negundo* leaf extracts reduces Triton-X-100 induced hemolysis at increasing concentration. Values are the average of triplicates and expressed as mean ± standard deviation

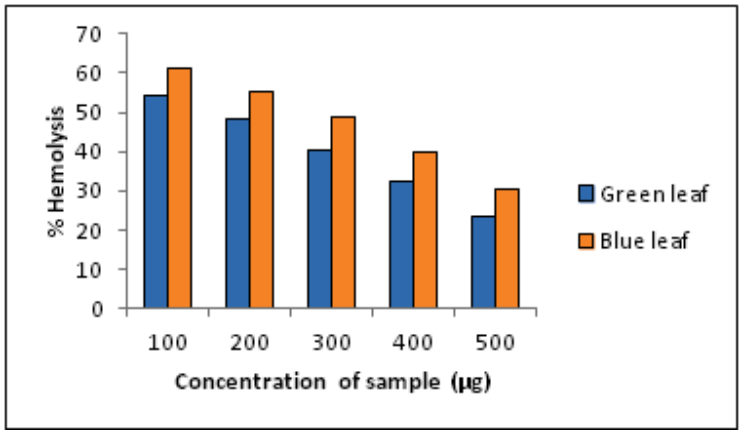

Figure 9A: Reduction in the *Naja naja* venom induced Hemolysis is observed upon treatment of *V.negundo* leaf extracts. Values are the average of triplicates and expressed as mean ± standard deviation

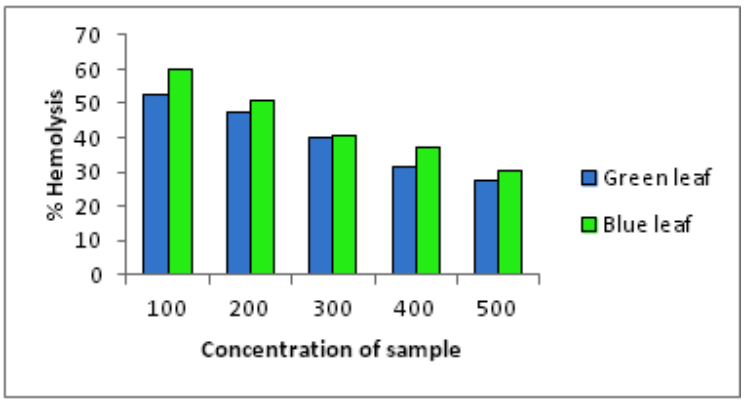

Figure 9B: Reduction in the *Daboia russelli* venom induced Hemolysis is observed upon treatment of *V.negundo* leaf extracts. Values are the average of triplicates and expressed as mean ± standard deviation

***In-vitro* venom neutralization**

Inhibition of Venom Induced Hemolysis by *Vitex negundo*

hydroethanolic extract of both blue and green leaves inhibited cobra and russell's viper venom induced hemolysis which was found to be indirectly proportional to the concentration of sample extract. The highest inhibitory effect against cobra venom induced RBC lysis was found to be 61% and 54.2% for blue and green leaves respectively. 60% and 52.5% inhibition was observed against Russell's viper induced RBC lysis when treated with blue and green leaves respectively. In both venom types, blue leaves exhibited maximum inhibition when compared with green leaves (Figure 9A &9B).

Inhibition of venom Induced Proteolysis by *Vitex negundo*

Both blue and green leaves demonstrated effective inhibitory effect against proteolysis induced by cobra and russell's viper venom. The maximum activity of 78% (Blue) and 76.8% (Green) was observed at 500µg/ml concentration against cobra venom induced proteolysis. 66.4% and 63.4% inhibition was observed for both blue and green leaves (500µg/ml) against russell's viper venom induced proteolysis respectively (Figure 10A & 10B).

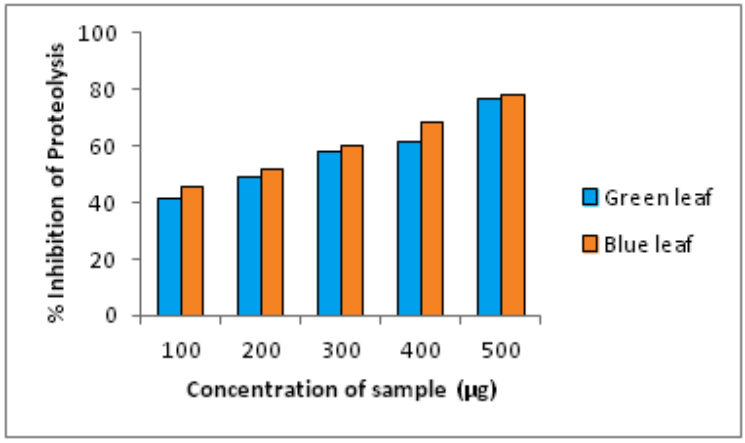

Figure 10A*: V.negundo* leaf extract inhibits *Naja naja* venom induced Proteolysis in concentration dependant manner. Values are the average of triplicates and expressed as mean ± standard deviation

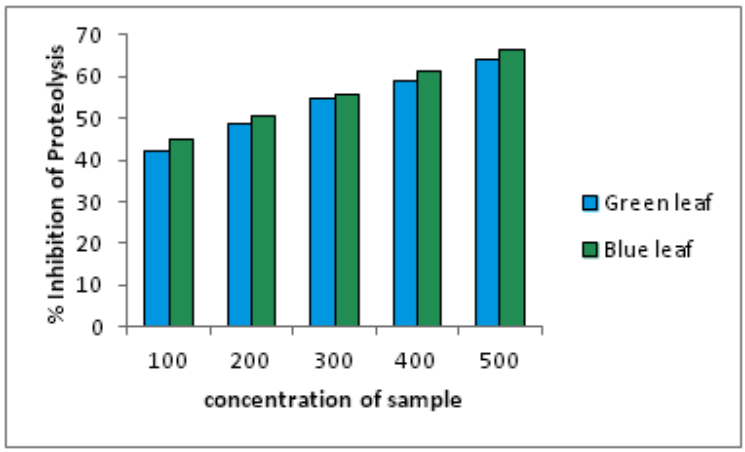

Figure 10B: *V.negundo* leaf *e*xtracts Inhibit *Daboia russelli* Venom Induced Proteolysis in concentration dependant manner. Values are the average of triplicates and expressed as mean ± standard deviation

Procoagulant Activity

The results of procoagulant activity caused by cobra and russell's viper venom with blue and green leaves of *Vitex negundo* are as follows: the cobra venom induced clotting in human citrated plasma within 35seconds and similarly russell's viper venom induced clotting within 32seconds and the minimum coagulant dose (MCD) was found to be 20µl and 15µl for cobra and russell's viper venom respectively. It was found that 1.6mg of blue leaf extract completely inhibited clotting induced by cobra and russell's viper venom in human citrated plasma. 1.8mg concentration was observed in case of green leaves against cobra and russell's viper venom. Thus, blue leaves were found to be more effective in inhibiting blood clotting caused by snake venom than green leaves.

Inhibition of Phospholipase A2 Activity

The results of Phospholipase A2 inhibition by both blue and green leaves of *Vitex negundo* against cobra and russell's viper venom are depicted in figure 11A & B. 10µl russell's viper and cobra venom were able to produce hemolytic halos of 11mm in agarose-erythrocytes gels. It was found that 1.0mg of Blue leaves and 1.2mg of Green leaves significantly reduced the diameter of hemolytic halo from 11mm to 6mm and 7mm respectively.

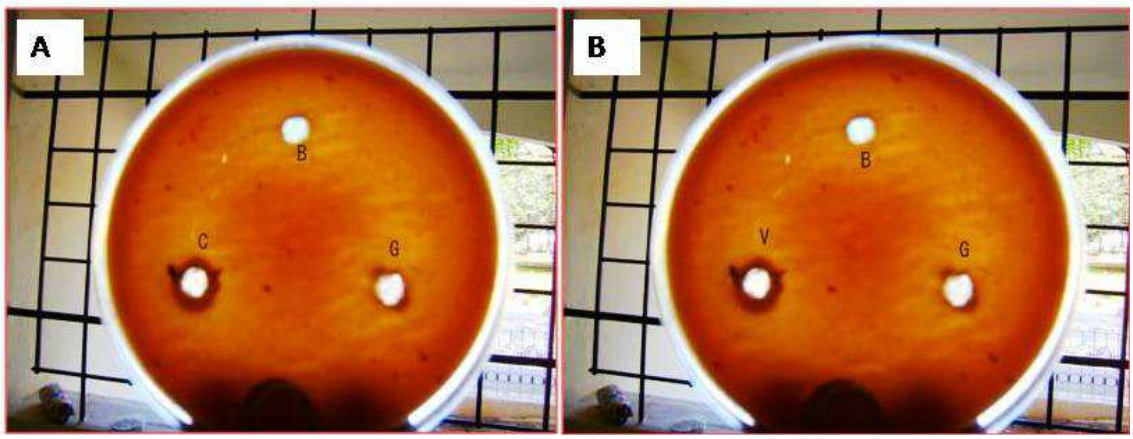

Figure 11: Inhibition of PLA_2 activity by blue and green leaves of *Vitex negundo* against (A) *Naja naja* venom and Daboia *russelli* (B) ; B-Blue leaf, G-Green leaf, C-Cobra venom alone, V-Viper venom alone

Inhibition of Fibrinolytic Activity

Snake venom contains a coagulant which acts by causing lysis of fibrinogen to fibrin monomers. This leads to fibrinogenalysis which in turn leads to blood clotting within seconds of snake bite. The results of inhibition of fibrinolytic activity by both Blue and Green leaves of *Vitex negundo* were depicted in figure 12. 10µl russell's viper and cobra venom produced fibrinolytic halos of 10mm in agarose gels containing human plasma. They were capable

of inhibiting fibrinolytic activity of both the venoms. It was found that 1.0mg of blue leaves and 1.2mg of green leaves significantly reduced the diameter of fibrinolytic halo from 10mm to 6mm and 7mm respectively.

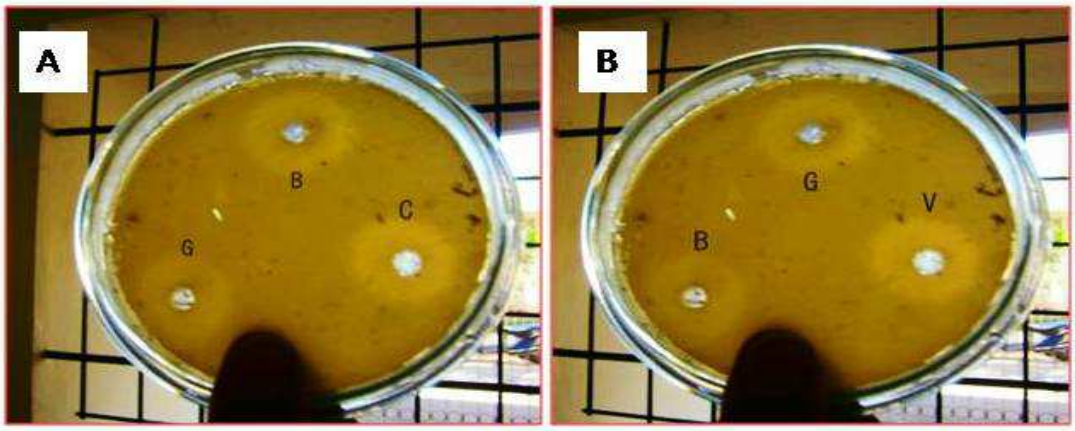

Figure 12: Inhibition of fibrinolytic activity by blue and green leaves of *Vitex negundo* against (A) *Naja naja* venom and *Daboia russelli* (B) B- Blue leaf, G-Green leaf, C-Cobra venom alone, V-Viper venom alone

DISCUSSION

Snake bite remains a major socio-medical problem worldwide. Reports claim that globally over one million humans are bitten annually by numerous snakes resulting in 70,000 deaths [35]. The major pathophysiological problems associated with snake bites generally starts with the systemic side effects like nausea, vomiting, headache, diarrhea, abdominal pain, and fall of blood pressure followed by late systemic effects, like neuromuscular blockage, respiratory, hemorrhage etc. sometimes, snake envenomation is associated with local side effects like pain, swelling and necrosis [36-37].

Polyvalent antiserum prepared from sheep and horses are effective for systemic envenoming after bite. They have their town limitations such as expensiveness and lack of availability for the rural patients to access [38]. Limitations related to antivenom serum therapy have made people to search for alternative medicines; especially from medicinal plants in the past few years. Apart from Indian traditional medicines, Chinese, Greeks and Egyptians are also motioned in the usage of folk and traditional medicinal plants in snake bite treatments [39]. Preliminary phytochemical screening revealed the presence of alkaloids, phenolics, flavanoids etc in the blue and green leaves. Although their specific roles were not investigated in this study, it has been reported that most active principles in leaves are frequently alkaloids, flavanoids and phenolics. These may be responsible for many of the pharmacological actions of the plant.

Free scavenging activity (DPPH, superoxide anion, hydroxyl, hydrogen peroxide) and reducing power activity of hydroethanolic extract of both blue and green leaf extract of *Vitex negundo* were investigated. Antioxidant properties of the pants play important role in neutralizing the snake venom [40-41]. It is evident from our study that extract of blue leaves showed maximum free radical scavenging potential though both the extract possessed dose dependant antioxidant activities in general. This result suggests that the possible free radical scavenging and antivenom potential might be due to the presence phytoconstituents present in the leaf extracts. Phytochemicals were analyzed for GC-MS investigation of both the leaf extracts after the qualitative phytochemical screening.

Snake venom contains enzymes called Phospholipase A2 (PLA2) which causes hemolysis of RBCs by acting on Human RBC (HRBC) membrane associated phospholipids liberating lysolecithin. Injury to RBC membrane in turn will render the cell more susceptible to secondary damage through free radicals. This study demonstrated the capability of *Vitex negundo* to stabilize RBC membrane and to prevent hemolysis. These results are in accordance with the results published by other studies [42-43]. Cobra and russell's viper venom contains several proteases that can lyze several important proteins and lead to erythrocyte membrane degradation. The proteolytic inhibition study showed the capability of plant extract to significantly inhibit protease activity, thereby protecting RBC membranes [44]. The coagulant in the venom directly activates factor X, which turns prothrombin into thrombin in the presence

of factor V and phospholipids. Thrombin in turn converts fibrinogen to fibrin clot, which forms the initial clot. The procoagulant activity demonstrated the ability of the plant extract to inhibit clotting caused by snake venom, probably by preventing the activation of Factor X [45]. Phospholipase A2 (PLA2) present in the venom acts by causing lysis of Phospholipids in RBC membranes. This leads to RBC membrane degradation and causes release of Arachidonic acids which in turn leads to Prostaglandin (PG) synthesis. PGs are the main cause of inflammation caused by snake venom. The phospholipase A2 inhibition activity suggests that the leaf extract contains some active constituents which might probably bind to the Phospholipase A2 enzyme, thus preventing it from binding to its substrate, thereby leading to its inhibition. These results are in par with previous studies [46]. In addition, snake venom coagulants induce lysis of fibrinogen and convert into fibrin monomers. This leads to fibrinogenalysis leading to blood clotting within seconds of snake bite. The fibrinolytic inhibition study demonstrated the ability of plant extract to inhibit fibrinogenolysis, probably by preventing conversion of fibrinogen to fibrin. These results are in accordance with other studies [45].

CONCLUSION

In conclusion, the present study proved the antivenom neutralization potential of the blue and green leaves of *Vitex negundo* against *Naja naja* (Indian cobra) and *Daboia russelli* (Russell's viper) venom. The presence of phytochemicals in both blue and green leaves support that this plant is valuable in ethnomedicine to treat snake bite. Blue leaves were found to exert potent antivenom potential when compared with green leaves of *V.negundo*. Blue leaves demonstrated maximum neutralizing capacity against cobra venom than russell's viper. The inhibitory activities of *V.negundo* against Indian cobra and russell's viper should be further confirmed by *in vivo* studies using animal models. Isolation of bioactive principles from the *V.negundo* leaves could lead to development of new natural alternative antidote for snake envenomation.

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