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EVALUATION OF PLATELET AUGMENTATION ACTIVITY OF *ADHATODA VASICA* AND ALKALOID FRACTION IN CYCLOPHOSPHAMIDE INDUCED THROMBOCYTOPENIC RATS

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ABSTRACT

Thrombocytopenia is a haematological feature associated with varied clinical conditions. Herb *Adhatoda vasica* (AV) has been reported in ayurveda for treatment of blood disorders and bleeding conditions and hence was selected to explore platelet augmenting activity. Quinazoline alkaloid vasicine present in AV possess array of medicinal properties and hence was quantified in chloroform (CEAV), methanol (MEAV), petroleum ether (PEAV) and hydro alcohol extracts (HEAV) using HPTLC. Among four different extracts, CEAV showed rich content of vasicine. Thus, alkaloid fraction (AF) was isolated from CEAV and presence of alkaloid was further confirmed by performing TLC. Thrombocytopenia was induced by administering cyclophosphamide (CPX) (50mg/kg, s.c) in wistar rats (180-230 gm) of either sex. CEAV (200 and 400 mg/kg daily) and AF (60 mg/kg daily) were administered orally to CPX induced thrombocytopenic rats for a period of 15 days. Complete blood count was monitored on 4th, 7th, 11th and 15th day of treatment. Bleeding and clotting time were determined on 15th day of treatment. CEAV and AF treatment significantly ($p<0.0001$) enhanced the platelet count and also attenuated prolonged bleeding and clotting time. CPX induced neutropenia and leukopenia were reversed on treatment with CEAV and AF. Levels of inflammatory markers TNF-α and IL-6 in serum were significantly decreased as compared to toxicant. Thus, the present study demonstrated platelet augmentation effect of AV. These observed effects might be due to immunomodulatory and anti inflammatory potentials of high content of vasicine found to be present in CEAV extract.

KEYWORDS: ***Thrombocytopenia, Adhatoda vasica, alkaloids, vasicine, platelets, Dengue virus***

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INTRODUCTION

Thrombocytopenia is a condition associated with decreased platelet count in blood below 1,50,000/ microL. It is a common clinical problem with numerous potential causes including decreased production of platelets in the bone marrow, increased destruction of platelets and alteration in distribution of platelets. According to WHO reports 2.5% of the normal population is affected by thrombocytopenia which may be hereditary or acquired[1]. Various disease status like chronic alcoholism, chronic liver disease and dengue hemorrhagic fever are main causes of thrombocytopenic conditions. Nowadays increasing preponderance of dengue fever associated with thrombocytopenia is of great concern[2]. It is estimated that there are currently more than 500,000 reported cases worldwide of dengue hemorrhagic fever and dengue shock syndrome[3]. Dengue virus (DENV) could directly or indirectly affect bone marrow progenitor cells by inhibiting their proliferative capacity of hematopoietic cells[4]. Hence, DENV can induce bone marrow suppression which leads to thrombocytopenia and associated coagulopathy. Currently no effective allopathic treatment is available to enhance platelet count. The only remedy available is platelet transfusion. Ayurveda claims platelet augmentation activity of various medicinal plants which can be used therapeutically to treat DENV induced thrombocytopenia if explored scientifically. Some of such medicinal plants are *Carica papaya* (Papaya), *Thinopyrum intermedium* (Wheatgrass), *Cucurbita pepo* (Pumpkin), *Sesamum indicum* (Sesame oil) etc. One such plant is *Adhatoda vasica* reported for its immunomodulatory effect and is also claimed in ayurveda for arresting bleeding during thrombocytopenic pupurea[5, 6]. Thus in this study plant *Adhatoda vasica* is selected to probe its platelet augmenting effect. *Justicia adhatoda* L.,synonym *Adhatoda vasica* (AV) is commonly known as Vasaka or Adulsa. AV is a medicinal plant native to Asia reported for its medicinal properties in ayurveda including expectorant, bronchodilator, antiseptic, antispasmodic, and anthelmentic etc. Literature survey reports presence of various phytoconstituents such as reducing sugars, anthraquinones, saponins, flavonoids, tannins , cardiac glycosides and quinazoline alkaloid namely 'vasicine' being a major phytoconstituent in AV. In addition to Vasicine , AV is also reported to be rich with other major alkaloids namely vasicinone and vasicinol with multiple therapeutic effects.[7] Among these alkaloids vasicine has been reported for anti-inflammatory, antioxidant, antitussive, antidiabetic, hepatoprotective and immunomodulatory effects[6]. Immunosupression and inflammation are major causes of thrombocytopenia. In the present study attempts have been made to explore platelet augmenting effect of both extract (CEAV) and alkaloid fraction (AF). Cyclophosphamide being an alkylating anticancer agent causes myelosupression leading to attenuation of maturation of megakaryocytes thereby inducing significant thrombocytopenia and hence this model was selected. In this study, attempts have been made to evaluate effect of CEAV and AF on complete blood cell count, bleeding time and clotting time in thrombocytopenic rats. In thrombocytopenic conditions usually circulating thrombopoietin concentration increases, in response to stimulate production of platelets from megakaryocytes[8,9]. Cytokines TNF –α and IL-6 play vital role in production of thrombopoietin[10,11] hence their levels have been also estimated. Thus the present research aims to assess platelet augmentation effect and probable modes of action of plant *Adhatoda vasica* and isolated alkaloid fraction to produce this effect.

MATERIALS AND METHODS

Materials used

Cyclophosphamide Monohydrate was procured from Tokyo Chemical Industry Co.Ltd. Vasicine was procured as a gift sample from ICT, Mumbai. *Adhatoda vasica* leaves were collected from Konkan areas. EDTA vials were procured from Labtech Disposables, Ahmedabad, India. All reagents used were of analytical grade procured from Merck, Ltd Mumbai, India and SD Fine Chemicals Ltd, Mumbai, India.

Preliminary phytochemical screening

Phytochemical screening of extracts was performed by using battery of tests for qualitative analysis for detection of phytoconstituents like alkaloids, reducing sugars, saponins, flavonoids, glycosides, tannins, terpenoids, and cardiac glycosides[12].

Preparation of plant extract

The plant was authenticated at Blatter Herbarium, St. Xavier college, Dept. of Botany, after matching with the existing specimen (Specimen no: p 6394).The leaves of *Adhatoda vasica* were air dried, powdered mechanically and sieved (#20). Powdered plant material was extracted in a Soxhlet apparatus with Chloroform (AR), Methanol (AR), Petroleum Ether and 70% v/v Hydro alcohol individually to obtain four different extracts of *Adhatoda vasica* viz CEAV, MEAV, PEAV and HEAV respectively. All extracts were evaporated on water bath at 50-60°C and were stored in refrigerator at 2-8° C [13].

Identification, quantification and validation of vasicine by hptlc[13]

Content of vasicine in extracts CEAV, MEAV, PEAV and HEAV was identified and quantified using HPTLC. 10 mg of standard vasicine (90% purity) was dissolved in 10 ml of methanol to prepare a stock solution of 1 mg/ml. For each extract 500mg was dissolved in 10 ml of methanol to prepare a stock solution of 100 mg/ml. Standard vasicine (2µl) and extract solutions (2µl) were applied over pre coated silica gel 60 F254 plates (stationary phase) using 100 microlitre Halminton syringe with help of Linomat V applicator. Application distance between two spots was maintained at 8 mm. Plate was run in mobile phase Ethyl acetate: Methanol: Ammonia (40:10:1). Plate was placed into CAMAG twin trough chamber for saturation of 20 minutes. After drying and developing plates for 15minutes, plates were scanned using CAMAG TLC Scanner, 1720422 at different wavelengths 254nm and 291nm. Data was analysed using integrated software vision (CATS). The concentration of vasicine in the extracts was determined by comparing the area of chromatogram of extracts with the calibration curve of standard vasicine. Method of development was validated with CEAV extract using standard vasicine. Specificity, recovery and

reproducibility were considered for the validation of developed method.

Isolation of alkaloid fraction from CEAV [14]

10 gm of CEAV extract was dissolved completely in 100 ml of chloroform under sonication (SPECTRALAB) for 10 minutes. The solution was filtered to remove insoluble matters. Filtrate obtained was further partitioned in 250 ml separating funnel with 30 ml x 6 portions of each of 5% NaOH solution to remove coloured compounds such as polyphenolic moieties and chlorophyll compounds. After treatment with NaOH colour intensity of dark green organic layer was quenched to pale green colour. The chloroform layer was dried to collect alkaloidal fraction. Presence of alkaloids in isolated fraction was confirmed by performing thin layer chromatography. The alkaloid fraction dissolved in chloroform solvent was spotted on TLC and run in mobile phase Ethyl acetate: Methanol: Ammonia (40:10:1).The plates were observed under UV light (λ=254 nm).

Animals

Wistar rats of either sex weighing 180-230 gm were procured from Bharat Serum and vaccines Limited, Thane. Animals were housed in institute's animal house (CPCSEA registration no. 25/PO/ReBi/S/99/CPCSEA Dtd-10/03/1999) at room temperature 24ºC±1ºC, relative humidity of 65%±10 % RH and 12 hour light and dark cycle. Animals were fed with standard pellet diet (Amrut Laboratory animal feed manufactured by D. S. TRADING AND CO. Borivali (W), Mumbai) and water *ad libitum*. The protocol for animal experimentation was approved by IAEC (Protocol no-KMKCP/IAEC/05/2017). Approved work was performed as per guidelines of the Committee for the purpose of control and supervision of experimentation on animals (CPCSEA) formed by the act of the Indian parliament under the prevention of cruelty to animal act 1960.

Experimental design

After acclimatisation period for 6-7 days, Wistar rats (180-230 gm) of either sex were divided into 6 groups each containing 8 animals and were treated as follows:

Group 1 VC (Vehicle control): Rats were administered with vehicle (2ml/kg p.o.) daily for a period of 18 days.

Group 2 TC (Toxicant control): Rats were administered cyclophosphamide (50 mg/kg/day s.c) for initial three consecutive days followed with administration of vehicle (2ml/kg p.o.) daily for a period of later 15 days.

Group 3 (Standard CP): Rats were administered cyclophosphamide (50 mg/kg/day s.c) for initial three consecutive days followed by treatment with standard drug Caripill (1100mg/kg/day p.o.) for later 15 days period.

Group 4(CEAV 200): Rats were administered cyclophosphamide (50 mg/kg/day s.c) for initial three consecutive days followed by treatment with CEAV (200 mg/kg/day p.o) for later 15 days period.

Group 5(CEAV 400): Rats were administered cyclophosphamide (50 mg/kg/day s.c) for initial three consecutive days followed by treatment with CEAV (400 mg/kg/day p.o) for later 15 days period.

Group 6 (AF): Rats were administered cyclophosphamide (50 mg/kg/day s.c) for initial three consecutive days followed by treatment with AF (60 mg/kg/day p.o) for later 15 days period.

Blood was withdrawn periodically on 4th, 7th, 11th and 15th day of treatment in EDTA vials (UNILAB) from retro orbital plexus by subjecting animals to light anaesthesia. Complete blood count was determined by using auto haematology analyzer (MINDRAY BC 3000 plus) for each time interval. Bleeding and clotting time were determined on 15th day of treatment by Duke's and capillary method respectively. On 15th day of treatment, serum was separated from collected blood by centrifugation at 3000 rpm for 10 minutes at 4˚C for the estimation of TNF-α and IL-6.

Haematological analysis

Complete blood count determination[15]

After blood withdrawal in EDTA vials, vials were gently shaken for two to three times and blood was processed under auto haematology analyzer machine (MINDRAY BC 3000 plus) within 6 hours of collection. 13 µl of blood was used for CBC estimation. Complete blood count parameters included Platelet, WBC, Neutrophil, Eosinophil, Lymphocyte, Monocyte, RBC Counts and Haemoglobin content.

Bleeding time determination[15]

Bleeding time was determined by Duke's method. The exposed tail tip was cleaned with distilled water and wiped with alcohol. By using sterile sharp surgical blade, about 2 mm incision was made on tail tip. On appearance of blood from incision stopwatch was started immediately to record bleeding time. At interval of every 30 seconds, bleeding tip was blotted on filter paper till no more blood spot appears on it. The time from appearance of first blood drop from incision to the disappearance of blood spot on filter paper was recorded as bleeding time.

Clotting time determination[15]

Blood was withdrawn into capillary tube by puncturing retro orbital plexus. Stopwatch was started to record clotting time on appearance of blood after puncturing retro orbital plexus. Two third volume of capillary glass tube was filled with blood and was then kept warm at body temperature by holding in between the palms of both the hands for 30 seconds. After 30 seconds, small portions of the blood filled capillary tube were gently broken at interval of 30 seconds until a thin thread of clotted blood appears between two broken pieces of capillary tube. Immediately on appearance of clotted blood thread recording of clotting time was stopped. The time interval between the appearances of first drop of blood from punctured retro orbital plexus till the appearance of thread of clotted blood between two broken pieces of capillary tube was recorded as clotting time.

Biochemical estimations[16]

On 15th day of treatment, blood was withdrawn through retro orbital plexus and centrifuged (Remi) at 3000 rpm for 10 minutes at 4˚C to obtain a clear serum. The separated serum was then used to estimate levels of TNF-α and IL-6 at absorbance 450 nm by ELISA kits

(Krishgen Biosystems) on Micro Plate Reader (Biotek ELx800).

STATISTICAL ANALYSIS

Results were analysed using One-Way ANOVA followed by Tukey Kramer post hoc multiple comparison test using Graphpad Prism 7.

RESULTS

Phytochemical constituents

The preliminary phytochemical analysis of powdered leaves showed presence of alkaloids, reducing sugars, anthraquinones, saponins, flavonoids and tannins whereas glycosides, tannins, terpenoids and cardiac glycosides were found to be absent (Table 1).

Identification and quantification studies of vasicine

In HPTLC method, mobile phase Ethyl acetate: methanol: ammonia (40:10:1) was used as a solvent system for identification and quantification of vasicine in extracts CEAV, MEAV, PEAV and HEAV. Vasicine was identified in all the extracts at R_f 0.40 which matches with the R_f of standard vasicine. In quantification studies content of quantity of Vasicine was found to be highest in CEAV (0.08%w/w) as compared to other extracts MEAV (0.04%w/w), PEAV (0.002%w/w) and HEAV (0.025%w/w). Thus, CEAV extract was selected to validate the developed analytical method using standard vasicine (Figure 1 and Figure 2).

Validation of developed analytical method using ceav

The developed analytical method was validated with CEAV using standard vasicine in terms of specificity, recovery and reproducibility. The linear relationship between peak area and amount of vasicine applied was found within the range of 250-900 ng/spot with correlation coefficient 0.999. There was no interference observed from mobile phase and diluent. Thus, this mobile phase was found to be specific for vasicine. The average of percentage recovery of CEAV was found to be 95.66%. The intra-day and inter-day day precision expressed as % CV indicate that the proposed method was precise and reproducible.

Thin layer chromatography

The CEAV extract was used for isolation of alkaloid fraction. TLC plate-impregnated with CEAV extract was developed in mobile phase containing Ethyl acetate: Methanol: Ammonia (40:10:1). The developed TLC plate, under UV light at 254 nm showed 6 bands out of which R_f value of first band (Figure 3) was matched with standard vasicine (R_f=0.4).

Haematological analysis

Platelet count

In toxicant control group, platelet count decreased significantly ($p<0.0001$) on 4^{th}, 7^{th}, 11^{th} and 15^{th} day of treatment as compared to vehicle control group whereas group treated with CEAV 200 and CEAV 400 showed significant ($p<0.0001$) increase in platelet count on 4^{th},7^{th},$11^{th}$and 15^{th} day of treatment as compared with toxicant group. Treatment with AF also showed a significant ($p<0.0001$) increase in platelet count on 7^{th}, 11^{th} and 15^{th} day of treatment as compared to toxicant control group (Figure 4).

Wbc count

In toxicant control group, significant ($p<0.0001$) decrease in WBC count was observed on 4^{th},7^{th},11^{th} and 15^{th} day of treatment as compared to vehicle control group. On treatment with CEAV 200 and 400 significant ($p<0.0001$) increase in WBC count was observed on 7^{th}, 11^{th} and 15^{th} day as compared to toxicant control. Treatment with AF showed a significant increase ($p<0.0001$) in WBC count as compared with toxicant group (Figure 5).

Neutrophil count

On administration of cyclophosphamide for three consecutive days significant ($p<0.0001$) neutropenia was observed in all groups. On treatment with CEAV 200($p<0.01$), CEAV 400 ($p<0.0001$), and AF ($p<0.01$) significant increase in neutrophil count was observed as compared to toxicant control (Figure 6).

Monocyte count

In toxicant control group, no significant alteration in levels of monocyte count was observed as compared with vehicle group. Treatment with Caripill, CEAV 200, CEAV 400 and AF did not produce any significant alterations in levels of monocyte count as compared to toxicant control group (Figure 7).

Eosinophil count

In toxicant control group, no significant alteration in levels of eosinophil count was observed as compared with vehicle group. Treatment with Caripill, CEAV 200, CEAV 400 and AF did not produce any significant alterations in levels of eosinophil count as compared to toxicant control group (Figure 8).

Lymphocyte count

In toxicant control group, no significant alteration in levels of lymphocyte count was observed as compared with vehicle group. Treatment with Caripill, CEAV 200, CEAV 400 and AF did not produce any significant alterations in levels of lymphocyte count as compared to toxicant control group (Figure 9).

Rbc count

In toxicant control group, RBC count was decreased significantly ($p<0.0001$) on 4^{th}, 7^{th}, 11^{th}, and 15^{th} day of treatment as compared to vehicle control group. On treatment with CEAV 200, CEAV 400 and AF significant ($p<0.0001$) increase in RBC count was observed as compared to toxicant control group (Figure 10).

Haemoglobin (hb) content

In toxicant control group, Hb count was decreased significantly ($p<0.0001$) on 4^{th}, 7^{th}, 11^{th}, and 15^{th} day of treatment as compared to vehicle control group. Treatment with CEAV 200, CEAV 400 and AF significant ($p<0.0001$) increase in Hb count was observed as compared to toxicant control group (Figure 11).

Bleeding time

Bleeding time in toxicant control group was significantly ($p<0.0001$) increased to 518.12±6.11 seconds on 15^{th} day as compared to vehicle control. On treatment with

CEAV 200 and CEAV 400 bleeding time was significantly ($p<0.0001$) decreased to 470.62and 396.25seconds respectively as compared to toxicant group. Treatment with AF significantly ($p<0.05$) decreased bleeding time to 480.87 seconds as compared to toxicant group (Figure 12).

Clotting time

Clotting time in toxicant group was significantly ($p<0.0001$) increased to 238.75seconds on 15th day as compared to vehicle control. On treatment with CEAV 200 and CEAV 400, clotting time was significantly ($p< 0.0001$) decreased to 165.2 and 96.87seconds respectively as compared to toxicant group. Treatment with AF significantly ($p< 0.05$) decreased clotting time to 187.5 seconds as compared to toxicant group (Figure 13).

Estimation of inflammatory markers

Serum tnf-α

In toxicant control group, TNF-α level in serum was found to be significantly ($p<0.0001$) increased to 174.72 pg/ml on 15th day of treatment as compared to vehicle control group. Treatment with CP, CEAV 200 and CEAV 400 showed significant ($p<0.0001$) dose dependent decrease in TNF-α as compared with toxicant control group. Similarly on treatment with AF, TNF-α was significantly ($p<0.0001$) decreased as compared with toxicant control group (Figure 14).

Serum IL-6

In toxicant control group, IL-6 level in serum was found to be significantly ($p<0.0001$) increased to 231.97 pg/ml on 15th day as compared to vehicle control group. Treatment with CP, CEAV 200 and CEAV 400 showed significant ($p<0.0001$) dose dependent decrease in elevated IL-6 levels as compared with toxicant control group. Similarly on treatment with AF, serum IL-6 was found to be significantly ($p<0.0001$) decreased as compared with toxicant control group (Figure 15).

Table 1
Phytochemical screening

PHYTOCHEMICALS	TEST
Alkaloids	+ve
Glycosides	-ve
Flavonoids	+ve
Tannins	+ve
Reducing sugar	+ve
Phlobatannins	-ve
Terpenoids	-ve
Anthraquinones	+ve
Cardiac glycosides	-ve
Saponins	+ve

+ve indicates presence of phytoconstituent, -ve indicates absence of phytoconstituent

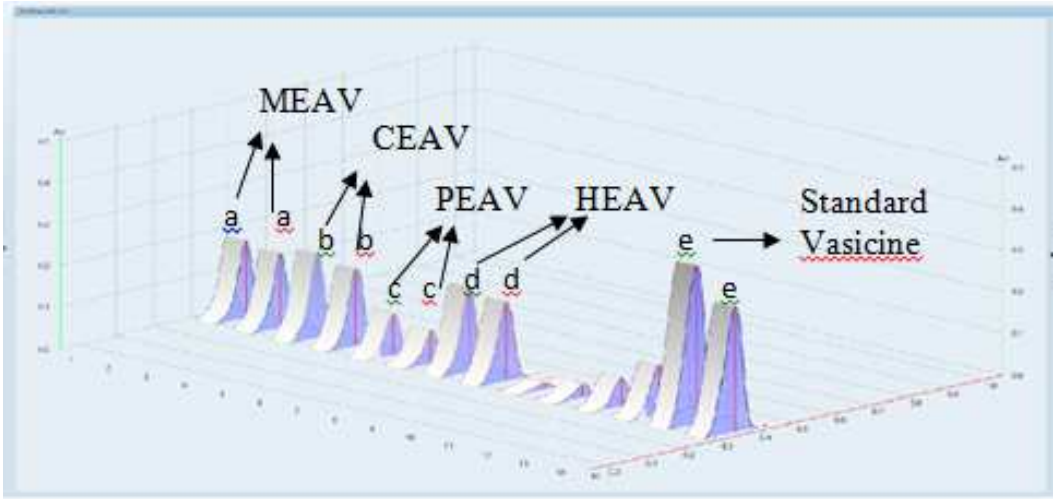
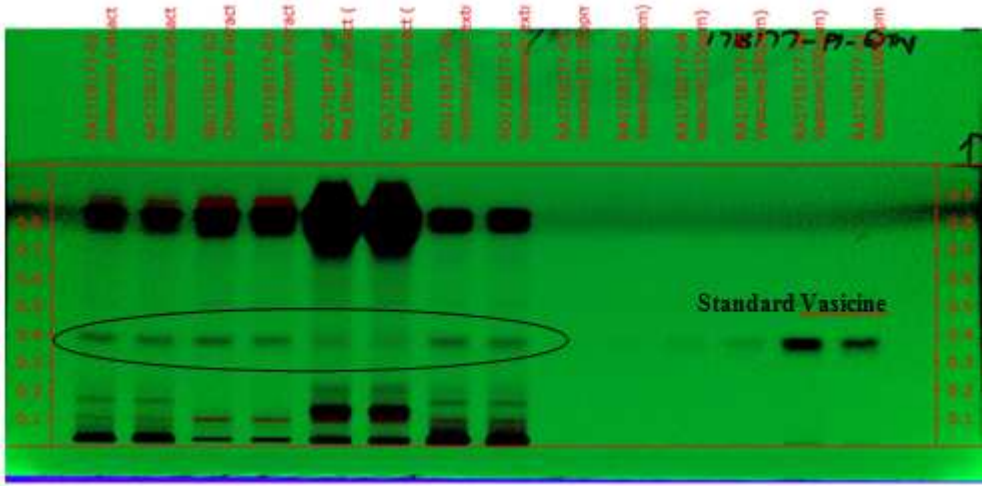


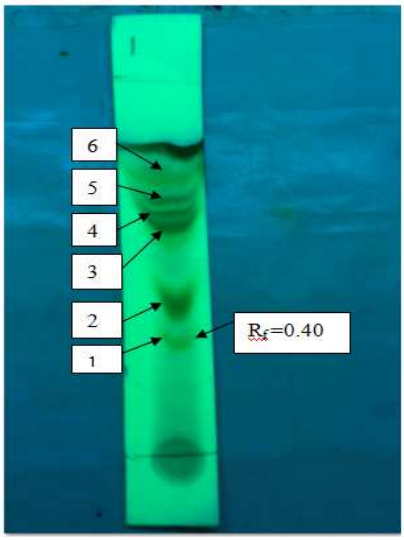
Figure illustrates densitogram of Standard vasicine (e) and vasicine in extracts MEAV (a), CEAV (b), PEAV(c), HEAV(d)

Figure 1
Identification of vasicine in CEAV, MEAV, PEAV, HEAV



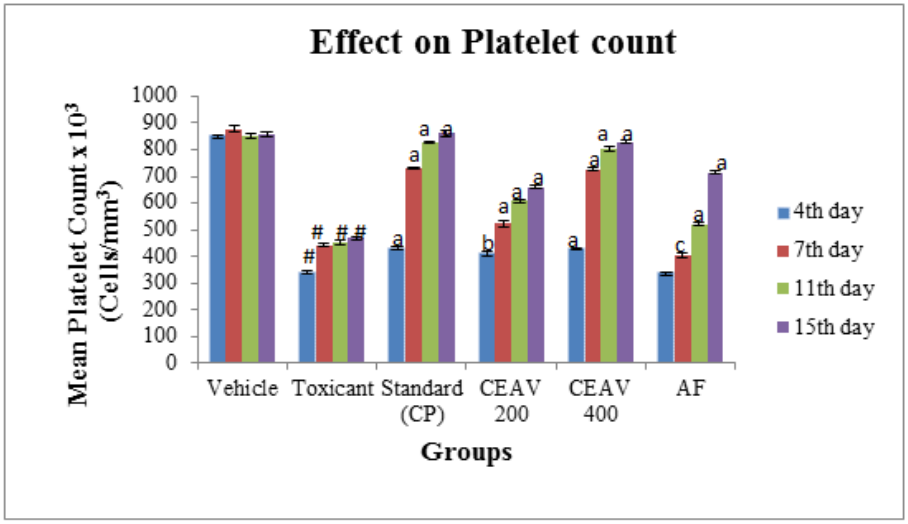
Above figure elucidates HPTLC fingerprinting of CEAV, MEAV, PEAV, HEAV extracts and standard vasicine. R_f of standard vasicine =0.40

Figure 2
Quantification of Vasicine in extracts



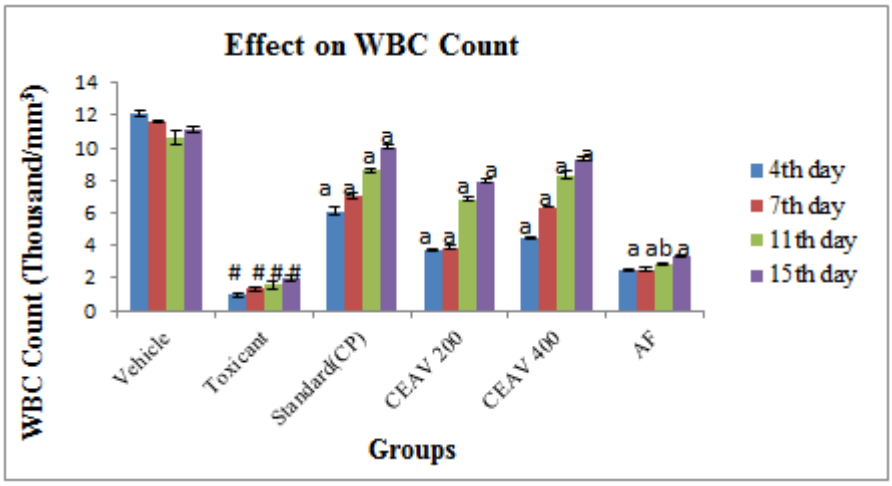
Numbers (1,2,3,4,5,6) in figure indicate bands of separated alkaloids Band No.1Vasicine at R_f 0.40

Figure 3
Thin layer chromatography of alkaloid fraction



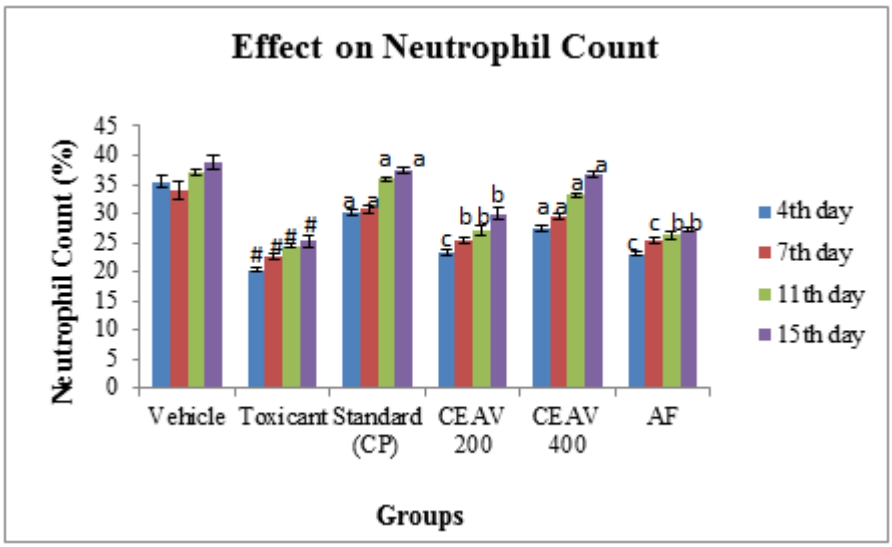
Values are expressed as Mean± SEM,(n=8).Statistically analysis was carried out by One-way ANOVAfollowed by Tukey-Kramer post hoc multiple comparison test. # P<0.0001 vs vehicle control and a P<0.0001, b P<0.01, c P<0.05 vs toxicant control. X axis represents Groups,Y axis representsMean platelet count x 10^3 (cells /mm^3)

Figure 4
Effect on Platelet Count



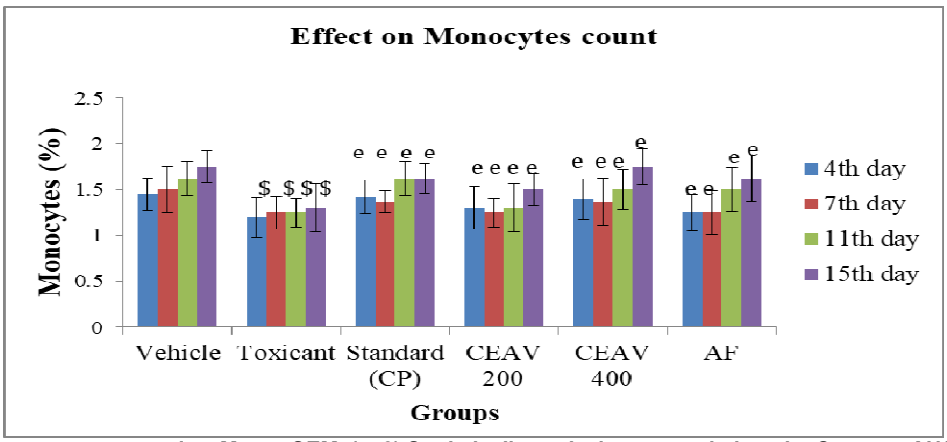
Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test.[#] P<0.0001 vs vehicle control and [a] P<0.0001,[b] P<0.01 vs toxicant control. X axis represents Groups,Y axis represents WBC count(thousand /mm^3)

Figure 5
Effect on WBC Count



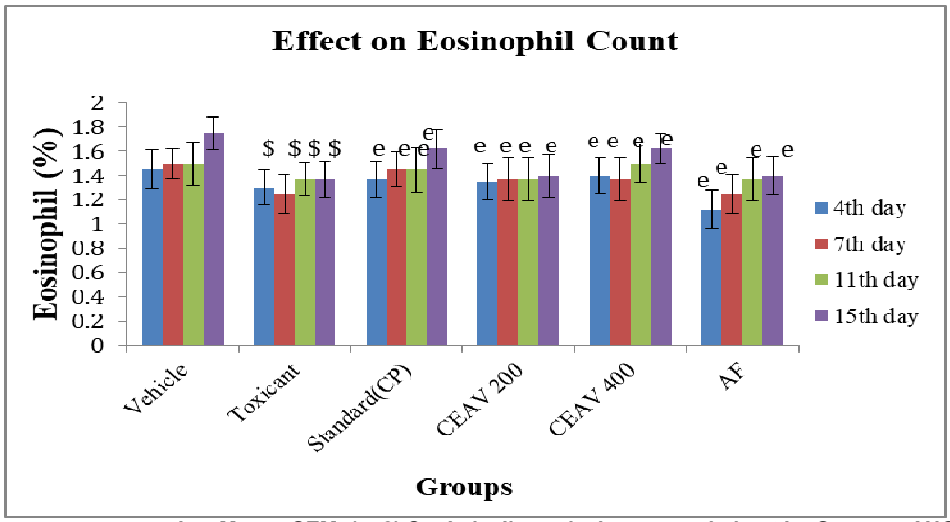
Values are expressed as Mean± SEM,(n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test.[#] P<0.0001 vs vehicle control and [a]P<0.0001,[b] P<0.01,[c] P<0.05 vs toxicant control. X axis represents Groups,Y axis represents Neutrophil count (%)

Figure 6
Effect on neutrophil count



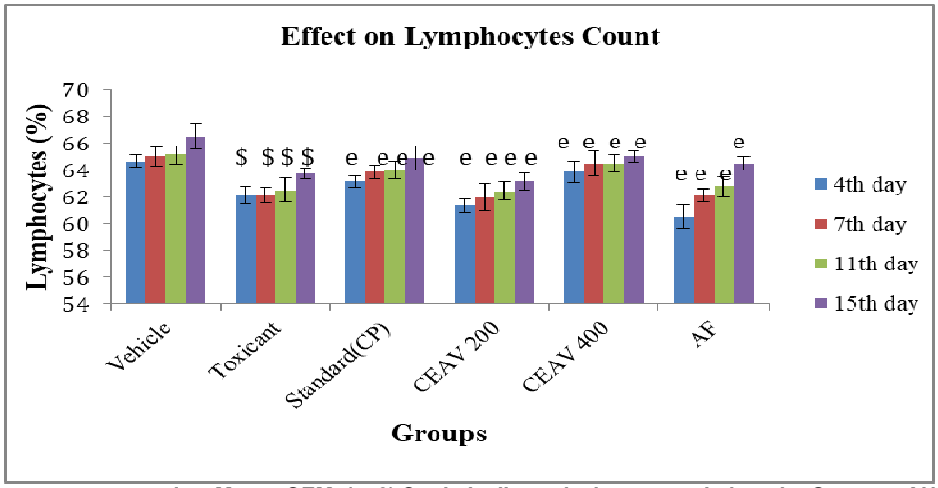
Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test [$]P>0.05 (NS)vs vehicle control and [e]P>0.05(NS) vs toxicant control.X axis represents Groups,Y axis represents Monocytes (%)

Figure 7
Effect on monocyte count



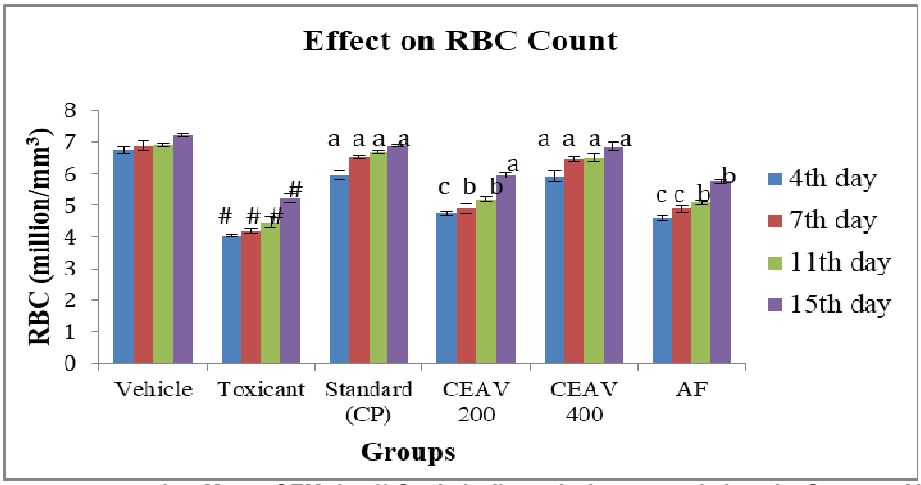
Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test $^{\$}$P>0.05 (NS) vs vehicle control and ^{e}P>0.05(NS) vs toxicant control.X axis represents Groups,Y axis represents Eosinophil (%)

Figure 8
Effect on eosinophil count



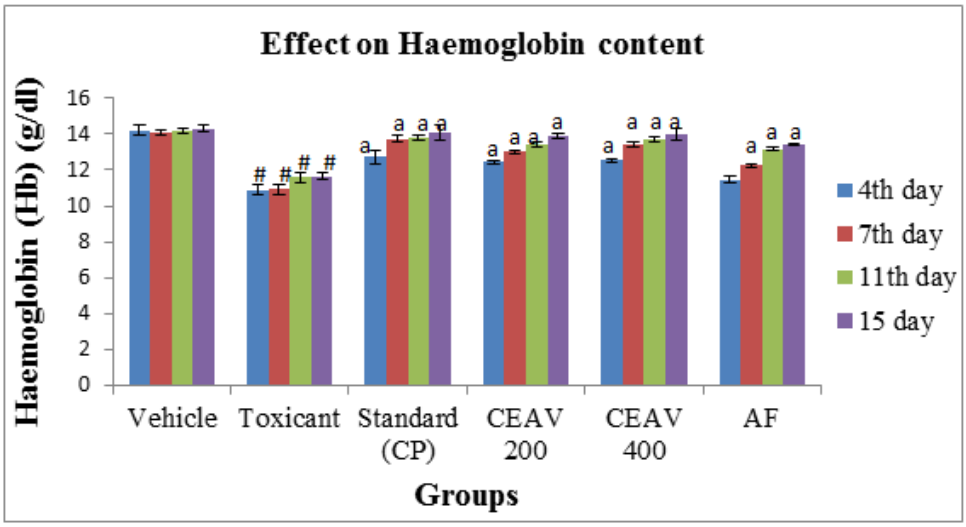
Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test $^{\$}$P>0.05 (NS) vs vehicle control and ^{e}P>0.05(NS) vs toxicant control. X axis represents Groups, Y axis represents Lymphocytes (%)

Figure 9
Effect on Lymphocyte count



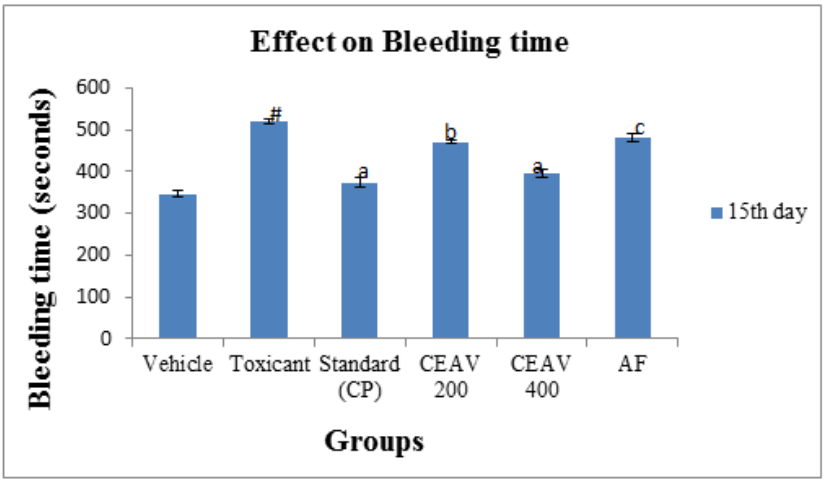
Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test.$^{\#}$P<0.0001 vs vehicle control and ^{a}P<0.0001,^{b} P<0.01, ^{c}P<0.05vs toxicant control. X axis represents Groups, Y axis represents RBC (million /mm^3)

Figure 10
Effect on RBC Count



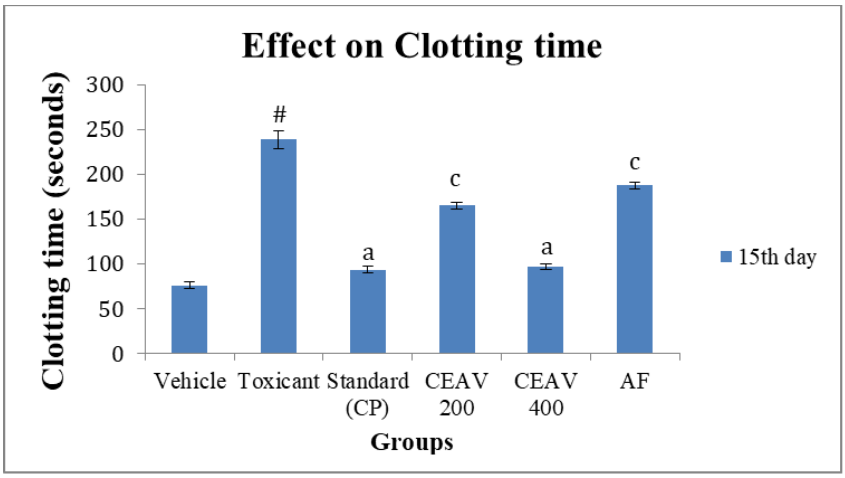
Values are expressed as Mean± SEM,(n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test.[#] P<0.0001 vs vehicle control and [a]P<0.0001vs toxicant control. X axis represents Groups, Y axis represents Mean Haemoglobin content (g/dL)

Figure 11
Effect on Haemoglobin content



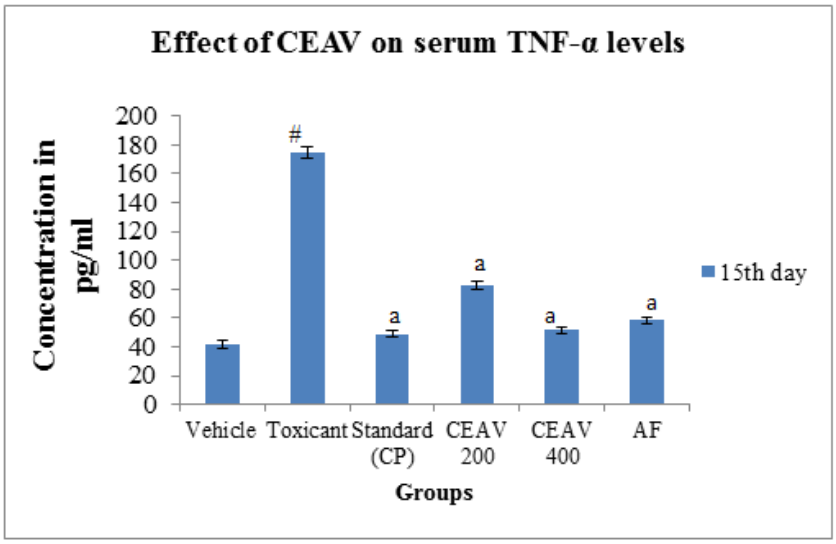
Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test.[#] P<0.0001 vs vehicle control and [a]P<0.0001,[b] P<0.01, [c]P<0.05 vs toxicant control. X axis represents Groups, Y axis represents Bleeding time in seconds.

Figure 12
Effect on Bleeding Time



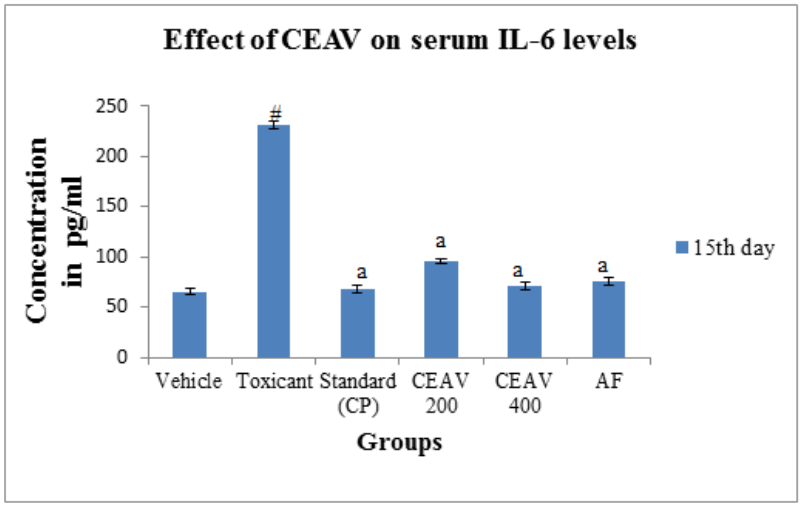
Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test.[#] P<0.0001 vs vehicle control and [a]P<0.0001, [c]P<0.05 vs toxicant control. X axis represents Groups, Y axis represents Clotting time in seconds

Figure13
Effect on Clotting Time



Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test. [#] *P<0.0001 vs vehicle control and* [a] *P<0.0001vs toxicant control. X axis represents Groups, Y axis represents Concentration(pg/ml)*

Figure 14
Effect on serum TNF-α levels



Values are expressed as Mean± SEM, (n=8).Statistically analysis was carried out by One-way ANOVA followed by Tukey-Kramer post hoc multiple comparison test. [#] *P<0.0001 vs vehicle control and* [a] *P<0.0001vs toxicant control. X axis represents Groups, Y axis represents Concentration (pg/ml)*

Figure 15
Effect on serum IL-6 levels

DISCUSSION

Platelets play a vital role in coagulation of blood. Many pathological conditions including aplastic anaemia, viral infections and liver diseases which affect platelet count leads to thrombocytopenia. One of the cardinal clinical features of dengue virus (DENV) infection is thrombocytopenia. In recent years, with increased incidences of DENV in Asian countries thrombocytopenia associated with DENV is of great concern. In dengue, thrombocytopenia is due to bone marrow suppression, impaired thrombopoiesis, and increased peripheral platelet destruction[17, 18]. Reported evidences of presence of DENV viral titres in platelets of infected patient indicates megakaryocytes as main target for dengue virus.[14,15] Conventional treatment used for thrombocytopenia induced by DENV involves platelet transfusion therapy, as no effective medication is available to augment platelet count. Ayurveda recommends use of medicinal plants for treating blood disorders. One such plant is *Adhatoda vasica* reported for immunomodulatory and anti-inflammatory effects and have also been therapeutically used to treat bleeding in idiopathic thrombocytopenia purpurea[19]. In this study, attempts have been made to explore platelet augmentation activity of leaves of AV against cyclophosphamide induced thrombocytopenia. Cyclophosphamide being an alkylating anticancer agent leads to myelosuppression causing attenuation of maturation of megakaryocytes resulting in thrombocytopenia associated with coagulopathy. Among all phytoconstituents present in AV, a quinazoline alkaloid vasicine comprises major one with a wide array of medicinal effects hence was quantified in this study. Highest content of alkaloid vasicine was found in Chloroform extract which guided isolation of alkaloid fraction (AF) from it. Extract CEAV and alkaloid fraction were then selected for *in vivo* evaluation of platelet augmentation activity. Metabolites of cyclophosphamide

induce oxidative DNA damage resulting in unrepaired DNA which ultimately leads to cell death through apoptosis. This effect leads to decreased blood cell count which was evident in our study from observed attenuation in platelet, WBC and neutrophil count in toxicant group. Dose dependent progressive increase in platelet count was observed over a period of 15 days on treatment with CEAV as well as alkaloid fraction which justifies its therapeutic potential in augmentation of platelet count. Cyclophosphamide induces thrombocytopenia by bone marrow suppression which is similar to that occuring during DENV[20] infection. Thus CEAV might prove therapeutically effective for treatment of thrombocytopenia during DENV infection. It was found that thrombocytopenia induced by cyclophosphamide has also resulted in increased bleeding and clotting time in toxicant control. However, treatment with CEAV and alkaloid fraction prevented the prolongation of bleeding and clotting time in dose dependent manner. This observed effect might be due to claimed therapeutic effect of AV in arresting bleeding during idiopathic thrombocytopenic purpurea[21,22] and also due to the availability of platelets for plug formation. Leukopenia and neutropenia were also observed on administration of cyclophosphamide which are common clinical features of dengue[23,24]. During dengue, neutrophil destruction associated with active release of chromatin fibres called neutrophil extracellular traps (NETs) are capable of trapping and killing microbes extracellularly. Evidences of viral infected platelets with NETs in blood samples of DENV infected patients have reported role of neutrophils in defence against DENV infection. In our study, WBC and neutrophil count were restored back to normal on treatment with CEAV indicating immunomodulatory potential of extract. Cyclophosphamide causes bone marrow suppression by inducing oxidative stress[25]. As Vasicine has been reported for its free radical scavenging activity[26] anti-oxidant potential of alkaloids present in CEAV thus might have resulted in myelo-protection against CPX causing restoration in levels of blood count including platelets. It has been reported that hormone thrombopoietin and platelet count are inversely related to each other as patients with thrombocytopenia secondary to myelosuppressive therapy have been reported with high levels of thrombopoietin. Correspondingly inflammatory mediators such as TNF-α and IL-6 play vital role in production of thrombopoietin [27] as TNF-α and IL-6 released during inflammation are circulated to liver to enhance thrombopoeitin which in turn stimulates megakaryocytes for production of platelets. In this study, significantly elevated levels of TNF-α and IL-6 observed in toxicant group are indication of induction of thrombocytopenia by CPX whereas attenuated levels of TNF-α and IL-6 on treatment with CEAV and AF is indication of restoration in levels of platelets. This effect might have occurred due to anti-inflammatory property of vasicine[27] leading to myeloprotection against CPX. It also eplores promising therapeutic role of both CEAV and AF to counteract inflammation associated with any other pathogenic conditions including DENV infection. The extent of platelet augmenting effect exhibited by extract CEAV was relatively greater than AF which may be due to presence of other phytoconstituents along with alkaloids in the extracts. Thus present study indicates that immunomodulatory, anti-oxidant and anti-inflammatory properties of alkaloids namely vasicine and other phytoconstituents present in CEAV might have led to observed myeloprotection against cyclophosphamide resulting in augmentation of platelet count. Increased platelet count on treatment with CEAV and AF in turn has also prevented coagulopathy associated with thrombocytopenia. The present study thus reveals promising therapeutic potential of plant *Adhatoda vasica* for treatment of thrombocytopenic conditions like those observed during dengue.

CONCLUSION

The present study suggests platelet augmenting effect of chloroform extract of *Adhatoda vasica* in thrombocytopenic conditions secondary to myelosuppressive therapy. This beneficial effect might be due to immunomodulatory, anti-inflammatory and antioxidant effects of alkaloids and other phytoconstituents present in CEAV. Thus, plant *Adhatoda vasica* could be an effective herbal remedy for the management of thrombocytopenia.

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AUTHORS CONTRIBUTION STATEMENT

Mrinal Sanaye conceived of the presented idea. Mrinal Sanaye and Zeba Dalwai investigated platelet augmentation effect of *Adhatoda vasica*. Both authors discussed the results and contributed to the final manuscript.

CONFLICT OF INTEREST

Conflict of interest declared none.

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