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Preclinical Evaluation of Thrombocytogenic Activity of Hydroalcoholic Extract of *Brassica oleracea*

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Abstract:

Brassica oleraceae, commonly known as broccoli, is a power house of nutrients for which they show potent pharmacological activities. In this research paper, we aimed to evaluate the thrombocytogenic activity of hydro alcoholic extract of *Brassica oleraceae*, as well as its effect on bleeding time by using two separate models. In this study the shed dried pieces of broccoli were extracted by 70% ethanol through double maceration and 3.8% yield was obtained.phytochemical screening performed with the extract revealed, presence of protein, flavonoids, steroids, alkaloids, amino acids, phenols, tannins, cardenolides and saponins. Albino wistar rats of either sex, weighing about 150 – 175 gm, were used for both the models. In cyclophosphamide induced thrombocytopenia rat model, all the animals were divided into 6 groups containing 6 animals in each group and treated with different drugs for a period of 14 days. On 7th, 10th and 14th day, standard group 1, 2, test group 1 (BOLD 250 mg/kg) and 2 (BOHD 500 mg/kg) showed significant increase in the platelet count as well as decrease in bleeding time when compared to toxicant control group. In chloramphenicol induced thrombocytopenia rat model, all the animals of 6 groups were treated with different drugs for a period of 6 days. On 6th day platelet counts of the animals were taken at an interval of 0, 1, 4 and 24 hours. At 24 hours standard group 1, 2, test group 1 (BOLD 250 mg/kg) and 2 (BOHD 500 mg/kg) showed a significant increase in the platelet count, as well as decreased bleeding time when compared to toxicant control group. It was concluded that the thrombocytogenic activity of *Brassica oleraceae* can be used to increase platelets count in thrombocytopenic disorder.

Keywords: Antioxidant, *Brassica oleraceae*, Chloramphenicol, Cyclophosphamide, Glucosinolates , Prednisolone, Thrombocytopenia.

1. INTRODUCTION

The history of herbal medicines is as old as our human civilization. Since ancient times plants are indispensible to human beings. Plants are assumed to be an inevitable part to protect our wellbeing, are nature's blessings to make malady free sound life. Traditional medicines obtained from medicinal plants are used by about 80% of people of developing countries due to presence of various bioactive components. Nowadays, as a result of our inquisitive nature we gathered a vast knowledge of therapeutic effects of different herbal plants. Thrombocyte or platelet, which is one type of blood cell, controls the blood loss by initiating the clotting mechanism. The average count of platelet is 150,000-450,000 per microliter. In case of blood loss, in pregnancy, presence of different illnesses like cancer, anemia, viral-bacterial infection, hepatitis C or HIV, because of different drugs (mostly chemotherapeutic), radiation, alcohol consumption or smoking may also reason increase breakdown of platelets. When the platelet count falls below 150,000 per microliter, the condition is stated as thrombocytopenia[1,2]. Severe internal bleeding can start when the platelets fall below 10000 per microliter. As we know that vitamin K plays an important role in blood clotting therefore we studied plants containing high proportion of vitamin K. *Brassic oleracea*, a cruciferous vegetable, is an excellent source of several vitamins (such as vitamin E, C, K etc), minerals (zinc, iron, selenium etc) along with its phytochemicals like glucosinolates and their byproducts, phenolics, antioxidant compounds[3,4]. It has potential pharmacological effects like anti-cancer[5], antioxidant[6], cardio protective[7], anti-diabetic[8], anti-asthmatic[9], anti-microbial activities[10], neuronal activities[11] along with goitrogenicity[12]. Due to its high vitamin K content, in this study we evaluate the effect of *Brassica oleracea* on platelet count in thrombocytopenia rat models.

2. MATERIALS AND METHODS

Fresh green papaya leaves and broccoli were collected from garden and local market respectively. Drugs and chemicals like ethanol, Prednisolone tablet (Wysolone 5 mg), Cyclophosphamide (Endoxan 50 mg), Chloramphenicol (Enteromycetin 500 mg tablet) were purchased from LOBA CHEME PVT. LTD., Wyeth Ledeted limited, Zydus oncoscience and Dey's medical stores respectively. Albino wistar rats of either sex, weighing about 150-175 gm, were procured from CPCSEA authorized breeder and observation executes according to the OECD guidelines.

2.1 Extraction:

The shed dried pieces of papaya leaves were extracted by double maceration method by using hydroalcoholic solution (50% v/v). The whole process of double maceration took 72 hrs, first step took 48 hrs and second one took 24 hrs. The extract was collected and kept under vacuum desiccators for evaporation. 4.7% yield was obtained.

The shed dried pieces of broccoli were extracted through double maceration process by using 70% ethanol for consecutive 72 hours. The extract was collected and kept under vacuum desiccators for evaporation. 3.8% yield was obtained.

2.2 Phytochemical Screening:

Hydroalcoholic extracts of Carica papaya leaves and broccoli, both were subjected to qualitative analysis for various phytochemical constituents like Alkaloids, proteins, amino acids, glycosides, tannins, flavonoids, steroids, carbohydrate, saponins, phenolic compounds[13].

2.3 Statistical analysis:

The experimental data were expressed as mean ± SEM for each treatment group. The significance of activity was assessed using a one-way analysis of variance followed by Dunnett's post-parametric test between the data of control and treated groups. *p<0.001 was considered statistically significant.

2.4 Experimental models:

2.4.1 Model 1: Cyclophosphamide induced thrombocytopenia in rat [14]

Cyclophosphamide is a well known anticancer drug. One of the major adverse effects of this anticancer drug therapy is bone marrow depression that results in granulocytopenia, thrombocytosis, agranulocytosis, aplastic anaemea. A total 14 days treatment was conducted. Animals were treated as follows:

Group 1: Normal group: animals of this group received only water, p.o. throughout the experiment period.

Group 2: Toxicant control group: animals of this group treated with cyclophosphamide 50 mg/kg s.c. for first three consecutive days.

Group 3: Standard group 1: animals of this group received prednisolone 200 mg/kg, p.o. for a period of thirteen days along with cyclophosphamide 50 mg/kg s.c. for the first three consecutive days.

Group 4: Standard group 2: animals of this group received papaya leaf extract 800 mg/kg, p.o. for a period of thirteen days along with cyclophosphamide 50 mg/kg s.c. for the first three consecutive days.

Group 5: test group 1: animals of this group received *Brassica oleraceae* low dose (BOLD) 250 mg/kg, p.o. for a period of thirteen days alongwith cyclophosphamide 50 mg/kg s.c. for the first three consecutive days.

Group 6: test group 2: animals of this group received *Brassica oleraceae* high dose (BOHD) 500 mg/kg, p.o. for a period of thirteen days alongwith cyclophosphamide 50 mg/kg s.c. for the first three consecutive days.

Blood samples were collected on day 1, 4, 7, 10 and 14 from the animals of each group and platelet count was done by using hemocytometer. On day 14 bleeding time of blood was determined.

2.4.2 Model 2: Chloramphenicol induced thrombocytopenia in rat

Chloramphenicol is obtained from Streptomyces venezuelea in 1947 and soon chemically synthesized for commercial purpose. It shows bacteriostatic as well as bacteriocidal activity, more active than tetracycline against H. influenze, S. typhi, by binding with 50s ribosome RNA complex, thus hinder the access of tRNA to the accepter site of amino acid incorporation, ultimately the formation of peptide bond gets inhibited in bacteria [15].

At high dose it usually inhibit mammalian mitochondrial protein synthesis, its toxic effects is responsible for bone marrow injury including aplastic anemia, thrombocytopenia, prolonged bleeding time and other hematological disorders. Chloramphenicol used to potentially inhibit the platelet protein synthesis, instigate to cell death and responsible for occasional thrombocytopenia.

A single dose of 50 mg/kg is enough to produce thrombocytopenia in the subject[16]. A drug with a serious side effects like thrombocytopenia, that also only after a single dose of administration definitely enhance the chance of establishing model having the motto to induce thrombocytopenia.

A total 6 days treatment period was conducted and animals were treated as follows:

Group 1: Normal group: animals of this group received only water, p.o. throughout the experiment period.

Group 2: Toxicant control group: animals of this grouped received chloramphenicol 50 mg/kg p.o. on the 6th day of experiment.

Group 3: Standard group 1: animals of this group received prednisolone 200 mg/kg, p.o. for consecutive 5 days followed by chloramphenicol 50 mg/kg p.o. on 6th day.

Group 4: Standard group 2 : animals of this group received papaya leaf extract 800 mg/kg, p.o. for consecutive 5 days followed by chloramphenicol 50 mg/kg p.o. on 6th day.

Group 5: test group 1: animals of this group received BOLD 250 mg/kg, p.o. for consecutive 5 days followed by chloramphenicol 50 mg/kg p.o. on 6th day.

Group 6: test group 2: animals of this group received BOHD 500 mg/kg, p.o. for consecutive 5 days followed by chloramphenicol 50 mg/kg p.o. on 6th day.

On 6th day, after completion of all treatment, blood samples were collected at 0, 1, 4 and 24 hours intervals and platelet count was done by using hemocytometer. Bleeding time was determined.

3. Result And Discussion

Preliminary phytochemical investigation of hydroalcoholic extract of *Carica papaya* leaves and broccoli is illustrated in table 1 & 2 which revealed the presence of alkaloids, proteins, tannins, flavonoids, steroids, carbohydrate and saponins in both the extract. Papaya leaf extract is devoid of glycoside whereas broccoli extract contains cardinolides.

TABLE 1: PRELIMINARY PHYTOCHEMICAL INVESTIGATION RESULTS OF PAPAYA LEAVE

Phytoconstituents	Results
Alkaloids	Positive
Proteins	Positive
Tannins	Positive
Carbohydrates	Positive
Glycosides	Negative
Flavonoids	Positive
Saponins	Positive
Steroids	Positive

TABLE 2: PRELIMINARY PHYTOCHEMICAL INVESTIGATION OF BROCCOLI EXTRACT

Phytoconstituents	Results
Proteins	Positive
Steroids	Positive
Flavonoids	Positive
Amino acids	Positive
Saponins	Negative
Cardinolides	Positive
Tannins and flavonoids	Positive
Alkaloid	Positive

Table 3 represent the data of platelet counts obtained from the Cyclophosphamide induced thrombocytopenia in rat model. Cyclophosphamide, a chemotherapeutic medication is used to treat lymphoma, multiple myeloma, leukemia, ovarian cancer, breast cancer, small scale lung cancer and sarcoma. The major side effect of cyclophosphamide includes bone marrow suppression followed by thrombocytopenia caused purpura, bruising and bleeding. In the cyclophosphamide induced thrombocytopenia in rat model thrombocytopenia was induced in normal rat by treating with cyclophosphamide 50 mg/kg s.c. for first three consecutive days. The thrombocytopenic animals were grouped and treated with prednisolone 200 mg/kg p.o., papaya 800 mg/kg p.o., BOLD 250 mg/kg p.o., BOHD 500 mg/kg p.o. as per their groups. On day 1, 4, 7, 10, 14 all the animals were subjected for platelet count. On day 4 prednisolone receiving group has shown increase in platelet count. On day 7 and 10 prednisolone as well as papaya, BOLD, BOHD has shown significant increase in platelet count when compared with toxicant control. On day 14 standard group 1 (prednisolne receiving group) and standard group 2 (papaya leaf extract receiving group) has shown average platelet count of 1246583 and 1231041 respectively. BOLD (test group 1) and BOHD (test group 2) has shown significant level of increasing in average platelet count that is 844905, 1025043 respectively when compared with negative control. From the above findings it can be stated that Broccoli in both lower and higher dose has shown thrombocytosis effect, and most promisingly in dose dependent manner. The effect of BOHD is somehow resembled with the effect of papaya. The graphical representation of this result is shown in figure 1.

Table 4 indicates the results obtained after determination of bleeding time. Evaluation of bleeding time is a strong parameter to establish the activity of thrombocyte. The control animals have shown the bleeding time 93 second. Cyclophosphamide retards the bleeding time on 180 second. Standard group 1 and Standard group 2 have accelerated the bleeding time at 78 and 84 second respectively. BOLD and BOHD has significantly decreases the bleeding time at 106 and 97 second respectively, while compared with the toxicant control. The effect of Brassica oleracea is much resembled with effect of standard. Figure 2 illustrates the graphical representation of bleeding time on 14th day.

TABLE 3: EFFECT OF DIFFERENT LEAF EXTRACTS ON PLATELET COUNTS IN RATS

Treatment group and dose (mg/kg)	Mean platelet count (cells per microliter) in time interval (day)				
	Day 1	Day 4	Day 7	Day 10	Day 14
Untreated control	933413 ± 23233	937004 ± 23395	932657 ± 19703	930211 ± 24525	934323 ± 18373
Toxicant control (cyclophosphamide) (50 mg/kg)	931726 ± 27320	726503± 28044	438906 ± 16093	386525 ± 31114	397071 ± 26522
Standard group 1 (prednisolone) (200 mg/kg)	940772 ± 22216	810513 ± 20488***	1231590 ± 21522***	1221446 ± 33709***	1246583 ± 8089***
Standard group 2 (papaya) (800 mg/kg)	926604 ± 12321	744593± 14333	1153742 ± 16522***	1198317 ± 8691***	1231041 ± 33041***
Test group 1 (BOLD) (250 mg/kg)	942511 ± 19621	720807 ± 17833	700826 ± 27005***	826336 ± 18041***	844905 ± 19826***
Test group 2 (BOHD) (500 mg/kg)	937026 ± 26018	728389 ± 18075	846551± 13825***	898474 ± 16801***	1025043 ± 11523***

All values are mean ± SEM, n = 6, ***p < 0.001 was considered statistically significant compared with untreated control group.

TABLE 4: BLEEDING TIME OF ALL GROUPS

Group	Bleeding time (sec)
Untreated control	93 ± 3.86
Toxicant control (cyclophosphamide)	180 ± 9.71
Standard group 1 (prednisolone)	78 ± 6.71***
Standard group 2 (papaya)	84 ± 4.46***
Test group 1 (BOLD)	106 ± 8.11***
Test group 2 (BOHD)	97 ± 5.26***

All values are mean ± SEM, n = 6, ***p < 0.001 was considered statistically significant compared with untreated control group.

TABLE 5: EFFECT OF DIFFERENT LEAF EXTRACTS ON PLATELET COUNTS IN RATS

All the values are mean ± SEM, n = 6, ***p<0.001 was considered statistically significant compared to untreated control group.

Treatment group and dose (mg/kg)	Mean platelet count (cells per microliter) in time interval (hour)			
	0 hour	1 hour	4 hours	24 hours
Untreated control	942914 ± 29266	927784 ± 14013	939997 ± 16926	943303 ± 18341
Toxicant control (chloramphenicol) (50 mg/kg)	926523 ± 32611	762914 ± 19517	676633 ± 8526	655140 ± 19010
Standard group 1 (prednisolone) (200 mg/kg)	1240536 ± 27014***	930771 ± 31016***	1090519 ± 25032***	1124733 ± 16042***
Standard group 2 (papaya) (800 mg/kg)	1191324 ± 21809***	888921 ± 16872***	913015 ± 18074***	1028314 ± 30302***
Test group 1 (BOLD) (250 mg/kg)	109013 ± 21036***	808313 ± 9054***	812907 ± 16807***	880542 ± 11817***
Test group 2 (BOHD) (500 mg/kg)	1181118 ± 25076***	826373 ± 13091***	842590 ± 13826***	924311 ± 16525***

TABLE 6: BLEEDING TIME OF ALL GROUPS

Group	Bleeding time (sec)
Untreated control	90 ± 4.71
Toxicant control (chloramphenicol)	175 ± 10.37
Standard group 1 (prednisolone)	80 ± 6.91***
Standard group 2 (papaya)	87 ± 3.83***
Test group 1 (BOLD)	104 ± 7.56***
Test group 2 (BOHD)	98 ± 5.59***

All values are mean ± SEM, n = 6, ***p < 0.001 was considered statistically significant compared with untreated control group.

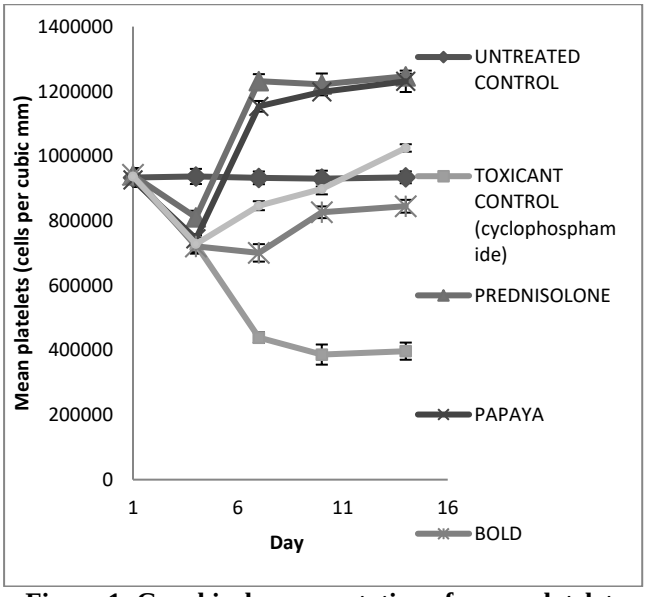

Figure 1: Graphical representation of mean platelet count with days

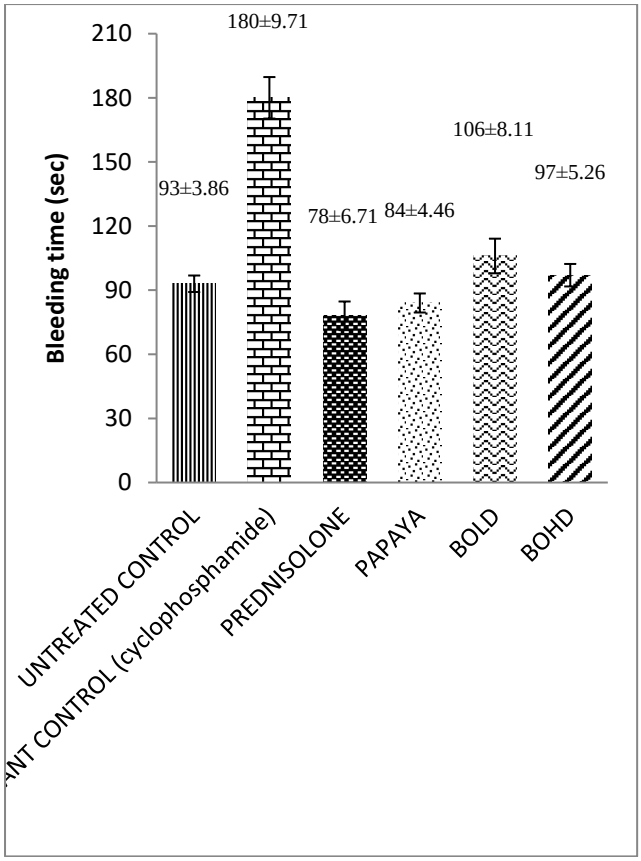

Figure 2: Graphical representation of bleeding time (sec) on 14th day

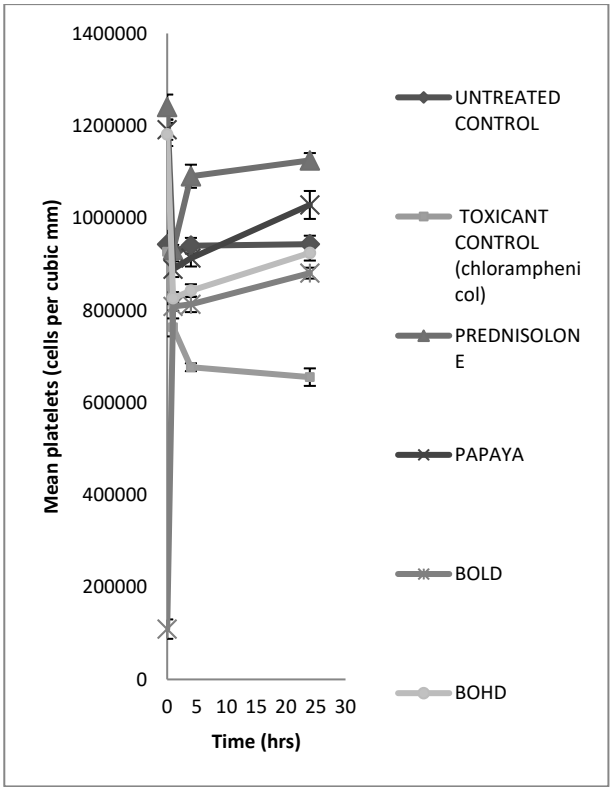

Figure 3: Graphical representation of mean platelet count with times (hrs)

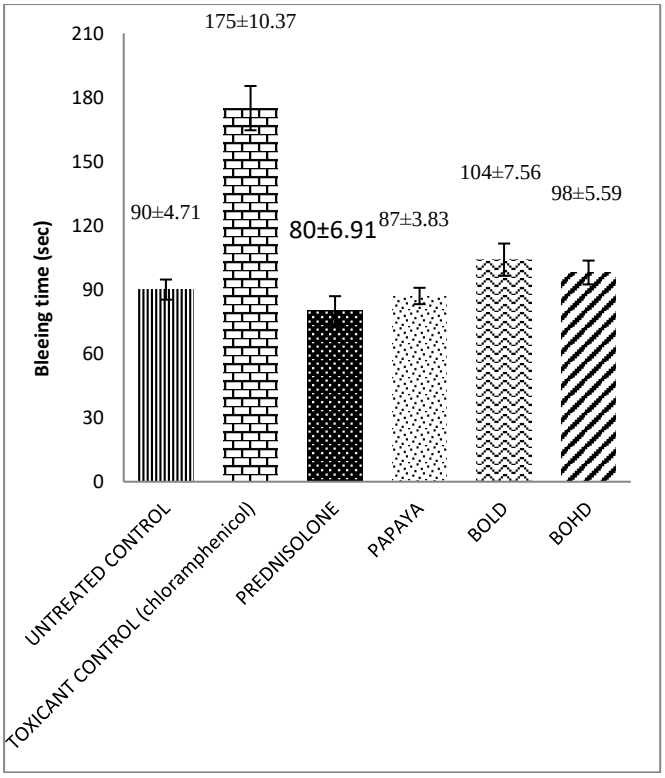

Figure 4: Graphical representation of bleeding time (sec) on 6th day

Most of the research paper regarding platelet count has been performed by cyclophosphamide induced thrombocytopenic model. In the present study another new model has been developed by focusing on the aim to overcome the errors that may arise by using a single model. Chloramphenicol, a broad-sprectrum antibiotic, binds with 50s subunit of ribosome-mRNA complex and inhibits the bacterial protein synthesis by interfering the transfer of elongated peptide chain from peptidyl site to newly attached aminoacyl-tRNA at the acceptor site that ultimately prevents the peptide bond formation. Simultaneously it causes the bone marrow suppression followed by thrombocytopenia as a side effect, that raises the chance to develop a new model where thrombocytopenia has to be induced in the animal to perform the further study.

The animals were grouped and received the treatment as per their groups for consecutive 5 days. All the groups except normal group has received a single dose of chloramphenicol 50 mg/kg on 6th day followed by platelet count on 0 hr, 1 hr, 4 hrs and 24 hrs. The data obtained from the experiment is illustrated in table 5 which clearly shows that BOLD, BOHD has given significant protection against the thrombocytopenic effect of chloramphenicol in dose dependent manner. At 24 hrs Prednisolone has shown 1124773 where as papaya has shown 1028314. At the same time BOLD and BOHD has shown the platelet count of 880542 and 9243111 respectively. All of these four groups shown significant level of thrombocytosis, regenerative activity while compared with toxicant control. The result obtained from this model is shown graphically in figure 3.

Table 6 portrays the result obtained after estimation of bleeding time. The control animals have shown the bleeding time 90 second. Chloramphencol retards the bleeding time on 175 second. Standard group 1 and Standard group 2 have accelerated the bleeding time at 80 and 87 second respectively. BOLD and BOHD has significantly decreases the bleeding time at 104 and 98 second respectively, while compared with the toxicant control. So it can be stated that the effect of Brassica oleracea is much resembled with effect of standard. The graphical representation of bleeding time is shown in figure 4.

From the above findings it is obvious that the chloramphenicol induced thrombocytopenic model has acted as a single dose acute model but still resembles with well-established cyclophophamide induced thrombocytopenic model. By using this model there is an attempt to overcome the limitations that may arise in case of single model dependent research. *Brassica oleracea* has been used as a clotting agent since long time in folk medicine and the same also has been proved scientifically[17]. The substance reduces the bleeding time definitely has a regenerative action on blood cell especially on platelet. In above two models *Brassica oleracea* has shown significant protective and generative effect on platelet count. Broccoli is an established antioxident agent and has supraoxide scavenging activity [18]. A substance reduces the free radicals load, definitely increases the viability of somatic and blood cells. At the same time it has a substance in the extract that acts as a coagulation factor consisting of vitamin K[19].Vitamin K plays an important role in clotting, including as a cofactor of carboxylation of glutamate residue in amino acid. These can be the viable mechanism to establish *Bassica oleracea* having a thrombocytogenic activity.

4 CONCLUSION

The present study was undertaken to evaluate thrombocytogenic activity of *Brassica oleracea* in thrombocytpopenia induced albino wister rat. The observation substantiates the use of *Brassica oleracea* in the treatment of dengue fever and to accelerate the clotting time in folklore system of medicine. Further study needed to observe its same activity to establish its molecular mechanistic pathway very clearly.

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