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SCREENING OF POLYHERBAL PLANT EXTRACTS AGAINST CARBOPLATIN INDUCED THROMBOCYTOPENIA IN MICE

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Dengue Hemorrhagic Fever (DHF), Thrombocytopenia, Carboplatin, Polyherbal formulation, *Carica papaya*, *Azadirachta indica*, *Andrographis paniculata*

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ABSTRACT: Thrombocytopenia, a condition in which platelet count decreases (<150-450 × 10^9/L), occurs as a result of clinical conditions such as viral infections or cancer or as a side effect of chemotherapy or it can be inherited, but if not treated on time it can be life threaten-ing. So, here we have evaluated the anti-thrombocytopenic potential of three plant extracts (aqueous extracts of *Carica papaya* and *Azadirachta indica*, methanolic extract of *Andrographis paniculata*) individually and their equal, unequal, half, and double proportionate combinations against carboplatin induced thrombocytopenia in mice. Thrombocytopenia has been induced successfully in all the treatment groups as well as in the disease control group, followed by a single intraperitoneal injection of carboplatin at the dose of 125 mg/kg of body weight. The extent of thrombocytopenia was the highest on day 7. Platelet count was significantly low in the disease control group ($p<0.0001$) compared to all other groups starting from day 7 to the end. All groups have responded to their respective treatment and have shown an increase in platelet count gradually with maximum effect on day 21, even more than the count on day 1. Our findings were further supported by bleeding time and clotting time. Both of these parameters remain almost unchanged in the normal control group but have been significantly prolonged ($p<0.0001$) in the disease control group, indicating an effect of fall in platelet count. When compared to the disease control group, all treatment groups have significantly less bleeding time ($p<0.0001$) and clotting time ($p<0.0001$).

INTRODUCTION: Thrombocytopenia is a clinical condition characterized by a decrease in platelet count (<150-450 × 10^9/L) [1, 2]. Platelets, also known as thrombocytes, are color-less blood cells, play an important role in the blood clotting process, and hence a fall in platelet count can hamper the process of blood clotting and prolong the bleeding time [2].

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Thrombocytopenia can be inherited, for example, Bernard-Soulier syndrome or acquired. In any circumstances, if platelet production is constantly decreased, which happen in the case of Myelo-fibrosis, Myelodysplastic syndromes, hematologic malignancies, severe vitamin/iron deficiency and use of drugs such as chemo-therapeutic agents, alcohol responsible for bone marrow damage or platelet consumption or destruction is constantly increased which happen in case of primary and secondary immune thrombocytopenia (ITP) caused by Canale-Smith syndrome, Post-trans fusional purpura, viral infections such as Dengue and its complications, Hepatitis C and sometimes it is drug-induced, or during pregnancy will eventually lead to thrombocytopenia [3].

Plant and plant-based herbal products have always been an important source of drug discovery and evaluation from the ancient times because of their high accessibility and lesser side effects [4]. The successful use of herbal products in the treatment of cancer has opened new gates in the field of herbal product screening and research. The World Health Organization (WHO) has advised people to interact and explore nature for safe, effective, and affordable remedies against diseases [5]. So to explore the rich Indian heritage and Ayurveda, Siddha, and Unani system of medicine, we have incorporated three different plant extracts into this protocol, which are aqueous extracts of papaya leaves and neem leaves as well as methanolic extract of the whole aerial body of kalmegh.

Carica papaya, commonly known as "Papaya" (family: Caricaceae) is a fast-growing shrub, reaches three to ten meters in height, grows best in warm, sunny sites, and having native to central America, now widely distributed to various tropical regions of the world [6]. Every part of the plant is known for its medicinal properties. The phytochemical analysis states the presence of various primary and secondary metabolites, which are flavonoids such as quercetin and kaempferol, terpenoids, alkaloids such as carpaine, phytosterols, carbohydrates, saponins, phenolics, tannins and glycosides, and being a lactiferous plant, it also contains papain, chymopapain, and other enzymes that aid the process of digestion [6, 7]. For years various parts of the plant are used as anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, anti-thrombocytopenic, anxiolytic and sedative, anti-hypertensive, hypoglycemic, free radical scavenger and anthelmintic, just a few [8, 9].

Another very useful and popular plant abundantly available in India is *Azadirachta indica*, commonly known as "Neem" belongs to the family Meliaceae. It is a large, tall, an evergreen tree, having origins in India but now cultivated in almost every continent of the world [10]. A decoction of seeds, leaf, bark, and fruit of neem has been a traditional folk medicine in India. Several studies have been done, and have documented its anti-bacterial, hepatoprotective, anti-fungal, anti-viral, anti-oxidant, anti-diabetic, and anti-malarial properties [11, 12] contributed to the presence of azadirachtin, nimbin, nimbolide, salanin, gedunin, nimbanene, nimbandiol, ascorbic acid flavonoids such as quercetin and β-sitosterol, saponins, triterpenoids and phenolic compounds [13].

Andrographis paniculata, commonly known as "Kalmegh" or "King of Bitters," belongs to the family Acanthaceae is an annual herbaceous plant. It is one of the very important constituents of many ayurvedic formulations [14]. It contains glycosides, mainly bitter ones, diterpenoids, and flavonoids with andrographolide as a principal constituent present in all parts of the plant [15]. It is a folklore medicine useful as anti-inflammatory, anti-malarial, anti-bacterial, cardiovascular protector, hepatoprotective, anti-obesity, anti-diabetic and anti-cancer [16].

Acute and chronic toxicity studies of these plants are very well reported [17-24]. However, there are innumerable herbs that may have the potential are waiting to be assessed; one should never forget that any of these, if not tested, can be fatal to human life. In the past few years, complementary and alternative medicine has gained tremendous popularity, so to arrive at any definite conclusion and going further in the field of herbal product research and development, we must evaluate their efficacy, safety and toxicity in animal models by *in-vitro* or *in-vivo* means.

MATERIALS AND METHODS:

Animals: Seventy-two healthy adult female swiss albino mice (Accuprec Research Labs. Pvt. Ltd., Ahmedabad, India) weighing 30–40 g were housed in an animal house facility under standard laboratory conditions (12:12 h light: dark cycle, relative humidity 30-70%, temperature 22 ± 3 °C, recorded thrice daily) in clean, sterilized poly-propylene cages (6 mice per cage). The mice were fed standard certified rat pellet feed (manufactured by Keval Sales Corporation, Vadodara) and drinking water treated by reverse osmosis, provided *ad libitum*. The experiment was carried out in strict accordance with guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. The procedures used in this protocol were reviewed and approved by the institutional animal ethics committee (ARL/PT/749/2019) before the initiation of the experiments.

Preparation of Plant Extracts:

Collection of Plant Material: Dry powder of *Carica papaya* leaves was obtained from Universal Agri Exports, Ahmedabad. Dry powder of *Andrographis paniculata* was obtained from Shri Shail herbs Pvt. Ltd, Nagpur. Neem leaves were collected from local areas of the Ahmedabad, dried under a shed and powdered to talc consistency in a mixer grinder and sieved. After collection, samples of collected plants and powders were sent to M.S. University, Baroda, for its identification and authentication. Our samples were evaluated for its identification and authenticity by the Department of Botany under specimen number Bot/21617/aut.

Extraction Procedure: Dried powder of *Andrographis paniculata* was extracted consecutively with absolute methanol (Merck, India) (1:5 w/v) for three days in dark condition. After three days, it was filtered (Whatman filter paper, grade 1) and evaporated under reduced pressure at 45 °C to dryness, stored at -4 °C [24]. Dry powders of *Azadirachta indica* and *Carica papaya* were subjected to aqueous extraction by cold maceration method [25]. Dry powders were extracted with chloroform and distilled water (2.5:97.5) in a ratio of (1:10) continuously for 3 days, and then it is filtered. The collected filtrate was then concentrated under reduced pressure at 45 °C and kept at -4 °C till use.

Induction of Thrombocytopenia: All the animals were divided into 12 groups (six animals each). Thrombocytopenia was induced in all groups except in the normal vehicle control group by a single intraperitoneal injection of carboplatin (Fresenius Kabi Oncology Ltd.) at the dose of 125 mg/kg of body weight [26].

Grouping and Treatment: At first, all the animals were weighed, and grouping was done based on their body weight, then thrombocytopenia was induced, and treatments were initiated by an oral route on the same day and continued for 21 days.

TABLE 1: GROUPING OF ANIMALS INTO CONTROL AND TREATMENT GROUPS

S. no.	Group	No. of animals	Dose concentration (mg/kg)
1	G1	6 Female	Normal Control (CMC 0.5%)
2	G2	6 Female	Disease Control (Carboplatin 125 mg/kg ip.)
3	G3	6 Female	T01 (300 mg/kg, p.o.) Equal proportion (33.33% of all the three extracts)
4	G4	6 Female	T02 (300 mg/kg, p.o.) (C:N:Kin ratio of 50,25,25%)
5	G5	6 Female	T03 (300 mg/kg, p.o.) (C:N:K in ratio of 25,50,25%)
6	G6	6 Female	T04 (300 mg/kg, p.o.) (C:N:K in ratio of 25,25,50%)
7	G7	6 Female	T05 (300 mg/kg, p.o.) (C:N:K in ratio of 70,15,15%)
8	G8	6 Female	T06 (300 mg/kg, p.o.) (C:N:K in ratio of 15,70,15%)
9	G9	6 Female	T07 (300 mg/kg, p.o.) (C:N:K in ratio of 15,15,70%)
10	G10	6 Female	T08 (500 mg/kg, p.o.) C extract
11	G11	6 Female	T09 (200 mg/kg, p.o.) N extract
12	G12	6 Female	T10 (200 mg/kg, p.o.) K extract

Where CMC = Carboxymethyl Cellulose, T = Treatment group. C= *Carica papaya* extract, N = *Azadirachta indica* (Neem) extract, K= *Andrographis paniculata* (Kalmegh) extract. In this protocol, with these permutations and combinations we have made the batch size of 12 gm, and all the extracts were weighed and combined accordingly

Blood Sampling and Parameter Measurement: Blood samples of all the animals were collected before the induction of thrombocytopenia and then on days 3, 7, 14, and 21 in K3 Ethylene-diaminetetraacetic acid (EDTA) containing tubes under light diethyl ether anesthesia from retro-orbital region, for the determination of Complete Blood Count (CBC), using an automated blood cell counter (Nihon kohden, MEK-6420p). At the same time, bleeding time and clotting time were also measured in seconds. The body weight of animals was recorded daily. All animals were observed daily for any signs of morbidity or mortality.

Statistical Analysis: It is carried out using the software Graphpad prism version 8. Data were recorded and noted as mean values +/- Standard Error of Mean (SEM). Data were analyzed by one-way, and two-way ANOVA followed by Dunnett's multiple comparison tests. Significance at $P<0.05$, 95% confidence interval was used for the analysis.

RESULTS:

Effect on Platelets: At the beginning of the experiment and before the induction of thrombo-cytopenia the average platelet count of normal control (NC) group was $(8.88 \pm 0.65) \times 10^5/\mu L$, and

disease control (DC) group was, (8.92 ± 0.68) × 10^5/µL. But after thrombocytopenia was induced, the platelet count in DC group was decreased compared to NC group on day 3, 7 (maximum decrease), 14, and 21 **Fig. 1**. In all treatment groups, we can see a significant change in total platelet count compared to the disease control group, indicating that our herbal extracts are acting positively alone as well as in combinations and the equal combination of all three extracts having the maximum increase, almost equal to or even greater than the normal control group. Data also suggests that the platelet count in all treatment groups is higher at the end of the study than it was on Day 1 **Table 2** and **Fig. 2**.

In this protocol, the highest fall in platelet count was observed on day 7 in DC group (3.94 ± 0.74) × 10^5/µL against (8.92 ± 0.68) × 10^5/µL on day 1, then it started increasing gradually (5.87 ± 0.68) × 10^5/µL on day14 and (6.27 ± 0.48) × 10^5/µL on day 21 (a significant decrease of 49.33%, 44.84%, and 32.75% respectively, p<0.0001) **Fig. 1**.

TABLE 2: AVERAGE PLATELET COUNT OF ALL GROUPS ON DAYS 1, 7, 14 AND 21

		Platelets (10^5/µL)											
Group	**Treatment**	**Day 1**			**Day 7**			**Day 14**			**Day 21**		
G1	Normal control	8.88	±	0.65	11.90	±	0.66	10.15	±	0.62	12.69	±	0.58
G2	Disease control	8.92	±	0.68	3.94	±	0.74	5.87	±	1.57	6.27	±	0.48
G3	Equal (33.33%)	7.85	±	0.52	4.48	±	0.97	8.55	±	0.49	13.62	±	0.98
G4	(C,N,K 50,25,25)	11.03	±	1.08	5.41	±	1.26	8.32	±	0.71	12.30	±	0.49
G5	(C,N,K, 25,50,25)	7.96	±	0.54	4.02	±	0.59	9.85	±	1.09	10.55	±	1.47
G6	(C,N,K, 25,25,50)	9.18	±	0.73	6.39	±	1.65	12.44	±	2.16	14.03	±	2.11
G7	(C,N,K, 70,15,15)	8.90	±	0.39	3.29	±	0.33	9.15	±	0.32	12.97	±	1.70
G8	(C,N,K, 15,70,15)	7.15	±	0.56	2.83	±	0.48	12.19	±	1.88	10.97	±	1.10
G9	(C,N,K, 15,15,70)	8.32	±	0.24	3.13	±	0.63	11.76	±	1.02	12.62	±	0.80
G10	Papaya 500 mg/kg	9.95	±	1.11	4.48	±	1.10	18.86	±	0.92	17.50	±	1.05
G11	Neem 200 mg/kg	7.39	±	1.17	2.56	±	0.40	11.01	±	2.01	13.50	±	1.69
G12	Kalmegh 200 mg/kg	8.07	±	0.56	4.60	±	0.96	9.29	±	1.34	13.71	±	0.68

Presented as Mean ± SEM

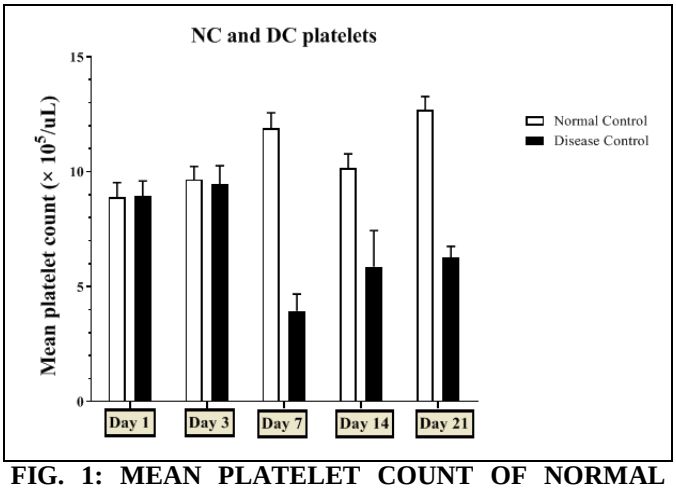

FIG. 1: MEAN PLATELET COUNT OF NORMAL CONTROL (NC) AND DISEASE CONTROL (DC) GROUP ON DAY 1, 3, 7, 14 AND 21 DEPICT THE EXTENT OF THROMBOCYTOPENIA. Values are expressed as Mean ± SEM. There is a significant decrease in platelet count on Day 7 and p<0.0001, Day 21 and p<0.0001 calculated using two way ANOVA and multiple t-test.

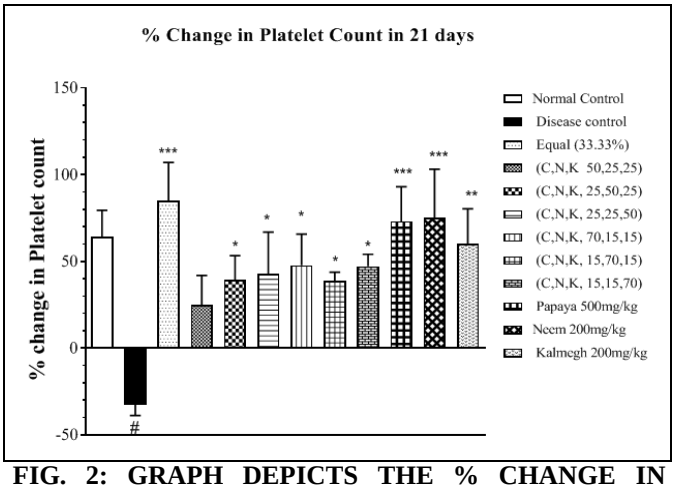

FIG. 2: GRAPH DEPICTS THE % CHANGE IN PLATELET COUNT IN ALL GROUPS IN 21 DAYS. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. Values are expressed as Mean ± S.E.M. (n = 6). Asterisk denotes treatment groups are statistically different from (#) Disease control, ***: p<0.001, **: p<0.01, *: p<0.05

Effect on Bleeding Time: The following data **Table 3** suggests that the bleeding time is not significantly altered in the NC group, but at the same time, it is significantly increased in the DC group. In case of treatment groups Bleeding time has been prolonged at the end of the study compared to day 1 **Table 3**, but it is significantly reduced when compared to the DC group **Fig. 3**.

TABLE 3: AVERAGE BLEEDING TIME OF ALL GROUPS ON DAYS 1, 7, 14, AND 21

Bleeding Time (sec)													
Group	**Treatment**	**Day 1**			**Day 7**			**Day 14**			**Day 21**		
G1	Normal control	21.00	±	1.2	19.83	±	1.30	20.33	±	0.88	19.40	±	0.94
G2	Disease control	19.83	±	1.2	46.00	±	1.98	52.17	±	1.14	54.33	±	2.05
G3	Equal (33.33%)	20.33	±	0.8	25.83	±	1.25	24.25	±	0.70	25.00	±	0.58
G4	(C,N,K 50,25,25)	19.00	±	0.8	29.60	±	0.47	29.75	±	0.51	29.75	±	0.51
G5	(C,N,K, 25,50,25)	19.83	±	1.3	28.83	±	0.70	27.40	±	0.85	26.50	±	0.85
G6	(C,N,K, 25,25,50)	20.83	±	1.1	30.80	±	1.45	25.00	±	0.33	27.33	±	1.03
G7	(C,N,K, 70,15,15)	20.83	±	0.9	30.40	±	1.10	21.25	±	0.84	27.00	±	1.29
G8	(C,N,K, 15,70,15)	20.67	±	1.0	19.67	±	0.88	24.33	±	0.85	28.67	±	0.62
G9	(C,N,K, 15,15,70)	20.67	±	1.4	30.67	±	1.69	27.00	±	0.58	30.00	±	0.58
G10	Papaya 500 mg/kg	20.00	±	1.0	23.00	±	1.95	22.25	±	1.12	25.25	±	0.91
G11	Neem 200 mg/kg	22.50	±	1.9	22.80	±	1.59	24.75	±	1.22	26.67	±	0.62
G12	Kalmegh 200 mg/kg	19.67	±	0.9	20.00	±	0.91	23.67	±	0.94	27.67	±	0.94

Presented as Mean ± SEM

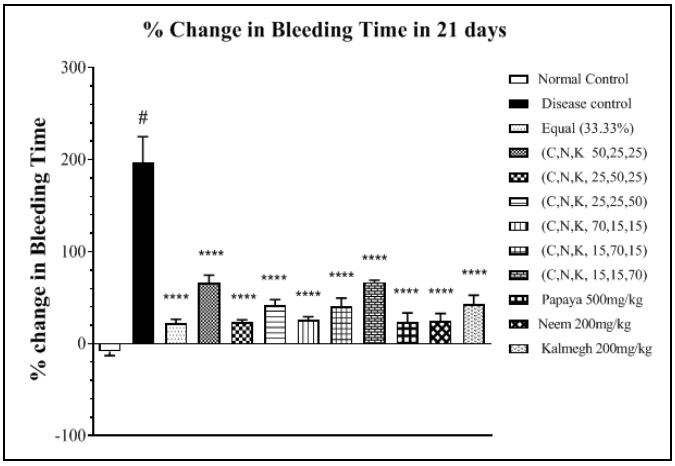

FIG. 3: GRAPH DEPICTS THE % CHANGE IN BLEEDING TIME IN ALL GROUPS IN 21 DAYS. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. Values are expressed as Mean ± S.E.M. (n = 6). Asterisk denotes treatment groups are statistically different from (#) Disease control, ****: p<0.0001

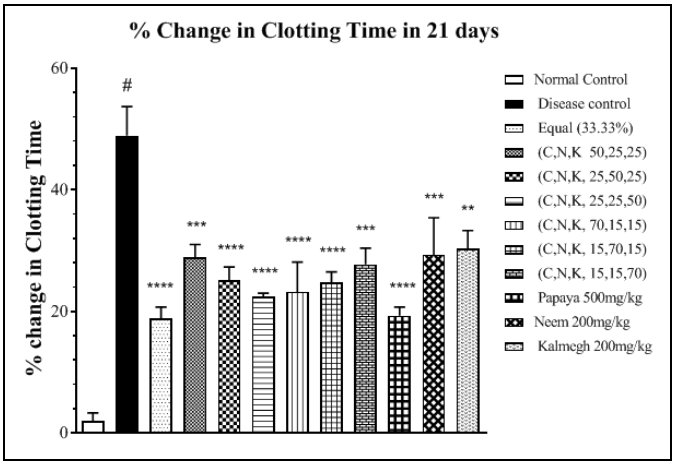

FIG. 4: GRAPH DEPICTS THE % CHANGE IN CLOTTING TIME IN ALL GROUPS IN 21 DAYS. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. Values are expressed as Mean ± S.E.M. (n=6). Asterisk denotes treatment groups are statistically different from (#) Disease control, ****: p<0.0001, ***: p<0.001, **: p<0.01

TABLE 4: AVERAGE CLOTTING TIME OF ALL GROUPS ON DAYS 1, 7, 14, AND 21

Clotting Time (sec)													
Group	**Treatment**	**Day 1**			**Day 7**			**Day 14**			**Day 21**		
G1	Normal Control	103.00	±	1.8	101.67	±	1.52	102.83	±	1.01	103.80	±	1.06
G2	Disease control	101.50	±	4.0	135.83	±	1.27	148.00	±	0.93	153.00	±	1.47
G3	Equal (33.33%)	103.00	±	2.3	122.83	±	2.30	128.75	±	2.27	123.75	±	1.68
G4	(C,N,K 50,25,25)	101.17	±	1.2	129.33	±	1.33	129.75	±	3.67	130.50	±	1.27
G5	(C,N,K, 25,50,25)	102.50	±	3.1	129.33	±	2.49	110.80	±	2.44	130.50	±	1.27
G6	(C,N,K, 25,25,50)	105.67	±	3.3	125.00	±	1.78	109.75	±	1.43	127.33	±	1.18
G7	(C,N,K, 70,15,15)	103.00	±	2.4	131.20	±	3.10	123.50	±	3.24	126.25	±	1.58
G8	(C,N,K, 15,70,15)	102.33	±	1.5	105.67	±	1.93	107.67	±	2.39	131.00	±	0.82
G9	(C,N,K, 15,15,70)	106.17	±	2.2	132.33	±	2.11	116.00	±	1.15	129.50	±	0.87
G10	Papaya 500 mg/kg	105.50	±	3.3	125.17	±	1.83	131.25	±	0.84	130.00	±	1.60
G11	Neem 200 mg/kg	99.83	±	1.4	131.00	±	1.00	131.00	±	1.20	130.67	±	4.17
G12	Kalmegh 200 mg/kg	105.67	±	1.9	126.40	±	1.37	134.67	±	1.65	137.00	±	0.41

Presented as Mean ± SEM

Effect on Clotting Time: There is no effect on clotting time in the NC group, it is almost the same on day 1 and day 21, but the DC group has shown a gradual increase in clotting time with the highest increase on day 21. Compared to day 1, clotting time in all treatment groups has been increased, but when compared to the DC group, all groups have shown a significant reduction in clotting time **Fig. 4**.

DISCUSSION: Carboplatin, a platinum-based chemotherapeutic agent which is a derivative of cisplatin and effective against several malignant tumors, can induce thrombocytopenia. It is used as it is considered to be less toxic in terms of causing nausea and vomiting, neurotoxicity, nephrotoxicity, ototoxicity, and highly myelotoxic [27]. Carboplatin induces thrombocytopenia by affecting the multilineage hemopoietic cells, which are mature and does not affect the stem cells [28, 29]. Thrombocytopenia was induced in all the groups except in the NC group. Treatment was initiated to prevent a fall in platelet count, and at the end of the study mean platelet count of all treatment groups was significantly higher than what it was on Day 1, complete reversion of thrombocytopenia. The highest effect was observed in group 3, which is an equal combination of all the three extracts (84.97%) followed by group 11 (Neem extract, 75.18%) and group 10 (papaya extract, 73.08%).

Bleeding time (BT) is the time required to stop the bleeding once the blood vessel is punctured. It is carried out to assess platelet function [30]. To stop bleeding, primary and secondary homeostasis will get activated in our body, characterized by weak platelet plug formation initially, which is a result of vasoconstriction, platelet adhesion, platelet activation, and platelet aggregation respectively, followed by the involvement of clotting factors to stabilize and strengthen the platelet plug. This clotting process is very much required to protect the body from excessive fluid loss [30, 31]. The time required for platelet plug formation is called clotting time (CT).

So, thrombocytopenia has a direct effect on both BT and CT, which is observed in our study. In NC group neither BT (Day 1: 21 ± 1.2 sec, Day 21:19.40 ± 0.94 sec) nor CT (Day 1: 103 ± 1.8 sec, Day 21:103.8 ± 1.06 sec) has changed, whereas in DC group both BT (Day 1: 19.83 ± 1.2 sec, Day 21: 54.33 ± 2.05 sec) and CT (Day 1:101.50 ± 4 sec, Day 21:153 ± 1.47 sec) has been significantly prolonged, $p<0.0001$. In DC group BT and CT have been prolonged by 197.2% and 48.9% in 21 days, indicating the extent of platelet malfunction. As all other groups have responded to all treatments, reflected as an increase in platelet count, the same results are observed in the case of BT and CT. However, in all treatment groups, both BT and CT are increased at the end of the study compared to what it was at the beginning, before the induction of thrombocytopenia, but when compared to the DC group, all groups have significantly reduced both BT and CT. The maximum effect was observed in group 3, which has shown an increase of only 22.4% in BT compared to 197.2% increase in DC group and 18.8% increase in CT compared to a 48.9% increase in DC group within 21 days **Table 3** and **4**.

Several case studies have reported the beneficial role of the papaya plant in the treatment of dengue [32, 33], but in our study, another two plants have also been emerged as potential candidates to be used against thrombocytopenia. All the three plants, when used alone, have shown positive effects on all the three major parameters, platelet count, bleeding time, and clotting time, but when they are used in combinations, especially in group 3 where all of them were used in equal proportions have shown the maximum efficacy as well as safety which is better than their individual use.

In India, thrombocytopenia has been a major component of both mild and severe cases of dengue resulting in morbidity and mortality. The severity, progression, and conversion of Dengue into its severe and life-threatening forms of Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) are directly proportional to the extent of thrombocytopenia [34]. In such cases and also in other cases where thrombocytopenia is the predominant factor responsible for morbidity or mortality, the search for plant and plant-based medicine can be an excellent remedy if used and authenticated properly.

CONCLUSION: In the present study it is observed that all three plant extracts, aqueous extracts of *Carica papaya* and *Azadirachta indica*, as well as methanolic extract of Andrographis paniculata, are having the potential to reverse the condition of thrombocytopenia alone as well as in combination. Our data suggest that when these three extracts are used in equal proportions, they give not only the best possible result but also the safest one. In the future, we may come out with a polyherbal formulation that can be used to treat any case where thrombocytopenia persists either as a

side effect of ongoing medicine, which happens mostly during chemotherapy or diseases such as dengue.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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