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ORIGINAL ARTICLE

Antithrombocytopenic Potential of Bioactivity Guided Fractions of Traditionally Used *Psidium guajava* Linn. Leaves in Busulfan Induced-Thrombocytopenic Rats

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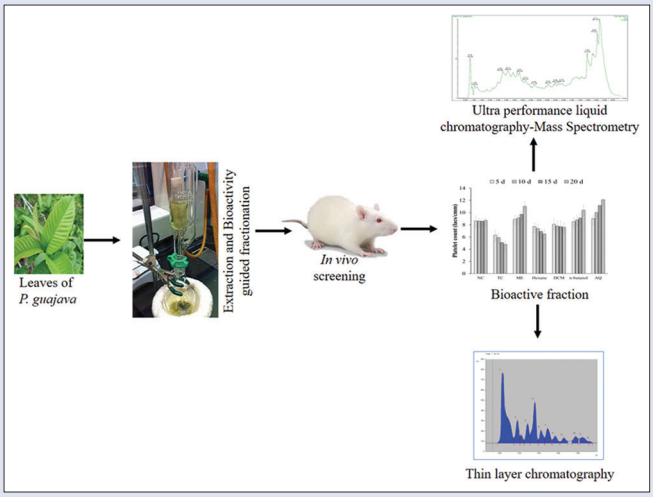
ABSTRACT

Background: *Psidium guajava* L. (family: Myrtaceae) is a traditionally used medicinal plant and possesses various therapeutic properties. This study was aimed to explore the antithrombocytopenic potential of bioactive fraction of *P. guajava* L. **Materials and Methodology:** Hydroalcoholic extract of dried leaves was prepared through soxhlation, and further, it was fractionated. Hydroalcoholic extract and its fractions were tested for antithrombocytopenic potential in busulfan-treated rats. Bioactive fractions were metabolically characterized through ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) and. **Results:** A total of 30.3% w/w hydroalcoholic extract was obtained from which 5.7%, 4.2%, 8.6%, and 11.8% w/w of n-hexane, dichloromethane, *n*-butanol, and aqueous fractions were collected, respectively. *n*-butanol and aqueous fractions were identified as the potent bioactive fractions as antithrombocytopenic agents. Using toluene:ethyl acetate:formic acid (6:3:1 v/v/v) as a solvent system in TLC, 12 and 9 metabolites were separated in *n*-butanol and aqueous fractions, respectively, whereas 11 and 12 metabolites were tentatively identified through UPLC-MS in aqueous and *n*-butanol fractions, respectively. Five metabolites (showing *m/z* values of 303.05, 302.5, 313.2, 573.51, and 341.32 eluted at 2.80, 3.81, 7.94, 8.55 and 8.69 min, respectively) were found in both aqueous and *n*-butanol fractions. **Conclusion:** Chromatographically characterized *n*-butanol and aqueous fractions of hydroalcoholic extract of *P. guajava* increased platelet count, and it can be further explored for the development of new phytopharmaceutical.

Key words: Antithrombocytopenic activity, guava leaves, *Psidium guajava*, thin-layer chromatography, ultra-performance liquid chromatography–mass spectrometry

SUMMARY

- Leaves of *Psidium guajava* were evaluated morphologically, microscopically, and chemically
- Leaves of *P. guajava* were subjected to hydroalcoholic extraction and bioactivity-guided fractionations
- *n*-butanol and aqueous fractions were found as the best antithrombocytopenic agent as compared to other fractions
- Bioactive fractions were metabolically characterized through ultra-performance liquid chromatography–mass spectrometry and TLC.



Abbreviations used: CMC: Carboxymethyl cellulose; DCM: Dichloromethane; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; MCV: Mean corpuscular volume; PCV: Packed cell volume; RBC: Red blood cell; R_f: Retardation factor; R_t: Retention time; TLC: Thin-layer chromatography; UPLC-MS: Ultra-performance liquid chromatography–mass spectroscopy; WHO: World Health Organization.

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INTRODUCTION

Thrombocytopenia is the result of falling in a number of platelets than normal range and is a common hematological disorder.[1] Based on platelet count (PC), thrombocytopenia is of three types, namely mild (100×10^9/L), moderate ($<100 \times 10^9$/L), and severe (50×10^9/L).[2] The physiological range of platelets in a normal healthy human is $150–500 \times 10^9$/L of blood. Typically, PCs $>500 \times 10^9$/L will not lead to any serious clinical problem, but intensive medical care is usually required for patients with PC $<30 \times 10^9$/L. Reduction in PC is due to its less production, rapid destruction, and sequestration.[3] Common causes of thrombocytopenia are viral infections, side effects of drugs, radiotherapy, vitamin deficiency, bone marrow infiltration by tumor, and bone marrow failure syndrome.[4]

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Dengue is the most evolving viral disease and has become a foremost health concern throughout the world, especially in India. The occurrence of dengue has increased dramatically across the globe in recent decade. An estimated, 390 million dengue infections occur every year globally which results in up to 25,000 deaths annually worldwide. The number of cases reported increased from 2.2 million in 2010 to 3.2 million in 2015.[5] It primarily affects our immune system, while the associated symptoms are a headache, mental disorders, hypertension, inflammation, liver enlargement, and thrombocytopenia with or without hemorrhage. Sometimes, people who are suffering from dengue may die due to its excessive severity, liver damage, and excessive bleeding,[4,6] and no specific treatment is available for dengue fever-induced thrombocytopenia.[7]

The association of thrombocytopenia with traditional remedies, nutritional supplements, foods, and beverages has been rarely described.[8] In today's scenario, there is an increasing interest in discovering new herbal medicine or phytopharmaceuticals which can be used for the management of thrombocytopenia and considered to be an important alternative to modern allopathic medicines.[9] One such example, *Psidium guajava* L. belongs to family Myrtaceae and has traditionally been used as a therapeutic agent for the treatment of hyperlipidemia,[10] diabetes mellitus,[11] cardiovascular diseases,[12] microbial infection,[13] cancer,[14] liver disorders,[15] parasitic infections,[16] anticestodal activity,[17] and antidiarrheal activity.[18] Recently, *P. guajava* leaves alone and in combination with other drugs (leaves of *Carica papaya*) have been used for the treatment of viral diseases and for balancing of PC.[19,20]

In the traditional system of medicines, different parts of *P. guajava*, especially leaves, have been used for various therapeutic purposes. Although several studies have been carried out to support traditional claim as antithrombocytopenic agent. Almost all these studies were performed on alcoholic or other organic extracts of *P. guajava*. In Indian traditional system of medicine, preferably aqueous or hydroalcoholic extract (1:1 v/v) or in powder form were used. In our study, an attempt has been made to develop a metabolically characterized bioactive fraction of *P. guajava* for the treatment of thrombocytopenia. The outcome of this study can be implemented for the development of new herbal drugs for the management of dengue fever and to reverse the thrombocytopenic conditions.

MATERIALS AND METHODOLOGY

Reagent and chemicals

Chemicals used in this study were of analytical grade and purchased from SD Fine Chemicals (India). Solvents were used for chromatographic analysis such as high-performance TLC and ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) analysis. Busulfan was purchased from Sigma-Aldrich (India) and used for induction of thrombocytopenia in rats. TLC plates (silica gel 60 F_{254}) were purchased from Merck (Darmstadt, Germany).

Plant materials

Fresh leaves of *P. guajava* were collected from Herbal Garden of Jamia Hamdard, Delhi, India. The collected plant materials were taxonomically identified and authenticated by Dr. Sunita Garg, Chief Scientist (RHMD) CSIR-NISCAIR, New Delhi, India. The authenticated sample has been stored in Bioactive Natural Product Laboratory for future reference (Voucher specimen no. NISCAIR/RHMD/Consult/2015/2889/82-2). Fresh leaves (10–12 cm in length) were selected for morphological observations by staining with safranin reagent. The specific diagnostic characters were studied by powdered microscopy.[21] Contemporarily, the World Health Organization (WHO) guidelines were used for evaluation of total ash, acid insoluble ash, water-soluble ash, loss on drying, extractive value, and pH value as physicochemical parameters.[22]

Preparation of extract and its fractionation

One hundred grams of powdered *P. guajava* leaves was extracted with 80% ethanol in Soxhlet extractor at 80°C for 16 h, and the extract was filtered. The filtrate was concentrated and dried to get the solid residue. The hydroalcoholic extract was further sonicated for approximately 15 min at 45°C for suspending in distilled water (1.0 g/10 mL). Aqueous suspension obtained was partitioned and polarity based fractionated using equal quantity of *n*-hexane, dichloromethane (DCM), *n*-butanol, and water (thrice each), separately. The prepared fractions were evaporated to dryness and stored in desiccators at room temperature for further use.

Quality control analysis by TLC

The precoated TLC silica gel GF_{254} plates (10 × 10 cm (Merck, Germany) were used for TLC analysis. The selected fractions were dissolved in respective solvents and diluted in high-performance liquid chromatography grade methanol. The prepared solution was filtered through 25-µ syringe filter. About 5.0 µL of the sample was applied on TLC plate using Linomat-V applicator (CAMAG, Switzerland). Toluene:ethyl acetate:formic acid (6:3:1 v/v/v) was used as a solvent system for maximum separation of metabolites. The applied plate was transferred to a CAMAG twin-trough glass tank with presaturated mobile phase for about 30 min at room temperature and 60% relative humidity. After 30 min, the plate was gently removed and air-dried. Further, the dried plate was scanned at 254 nm and 450 nm, respectively.[23] TLC fingerprinting of *n*-butanol and aqueous fractions of hydroalcoholic extract of *P. guajava* leaves was carried out for their quality control analysis and to determine the number of metabolites present in them. These methods were developed as per the International conference on Hormonization (ICH) guidelines, like other methods reported by the laboratory for quality control of herbal drugs and botanicals.[24]

Ultra-performance liquid chromatography–mass spectrometry

The prepared extract and fractions were dissolved in LC-MS grade methanol and filtered through 0.25-µ syringe filter and used for analysis. Waters ACQUITY UPLC™ system (Serial No #F09 UPB 920M; Model code #UPB; Waters Corp., MA, USA) equipped with an autosampler, a column manager, a binary solvent delivery system, and a tunable MS detector (Serial No #JAA 272; Synapt; Waters, Manchester, UK) was used for UPLC-MS analysis of extract and fractions. The sample was chromatographically separated in previously degassed mobile phase consisting of 0.5% v/v formic acid in water (A) and acetonitrile (B). The chromatographic separation was achieved using gradient elution mode (initially 100% A and hold for 5 min; further, decreased to 5% A in 20 min) and 0.4 mL/min was the flow rate of the mobile phase. Waters ACQUITY UPLC™ BEH C18 (100 mm × 2.1 mm × 1.7 µm) column was used as a stationary phase. The column manager and sample manager temperatures were set to 35°C ± 2°C and 25°C ± 0°C, respectively. Five microliters filtered sample diluted in methanol was injected with the help of auto-injector, and the maximum bearable pressure of the system was set to 15,000 psi.

The separated metabolites were detected by MS detector on a quadruple orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Q-TOF). The cone gas and nebulizer gas were set to 50 L/h and 500 L/h, respectively. The source temperature of the MS detector for ionization was set to 100°C. The cone voltage and capillary voltage were set to 40 kV and 3.0 kV, respectively. Argon gas was used for the collision of an ion at a pressure of 5.3×10^{-5} Torr. The Q-TOF Premier™ was operated in scan mode with resolution over 8500 mass

with 1.0-min scan time and 0.02-s interscan delay. Both UPLC and the mass detector were operated using MassLynx Version 4.1 built with the instrument. Based on the analyzed *m/z* value and literature survey, separated metabolites were identified.[25]

Evaluation of extract and fractions for antithrombocytopenic potential

The *in vivo* study was performed to carry out the antithrombocytopenic potential of *n*-butanol and aqueous fractions of hydroalcoholic extract of *P. guajava* after oral administration to female Wistar rat (150–250 g) as per the standard protocol. Animals were obtained from the Central Animal House Facility of Jamia Hamdard. The study protocol was approved (Approval Number: 915) by the Institutional Animal Ethics Committee, constituted by the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Animals were randomly distributed in seven groups ($n = 6$). Busulfan at a dose of 5.0 mg/kg was administered through subcutaneous route to rats for thrombocytopenia induction.[26] Busulfan was given to Wistar rats on days 1, 5, 10, and 15. On the same day of busulfan treatments, animals were treated with the test preparation for a period of 20 days. Group I, receiving 0.1% carboxymethyl cellulose (0.5 mL oral, once daily for 20 days), served as control; Group II to Group VII received busulfan at a respective dose. Group II, only received busulfan and served as toxic control. Throughout the experimental period, Group III received hydroalcoholic extract at a dose of 240 mg/kg; Group IV received n-hexane fraction (33 mg/kg); Group V received DCM fraction (27 mg/kg); Group VI fraction received *n*-butanol fraction (150 mg/kg); and Group VII received aqueous fraction (78 mg/kg). The dose of extract and fractions was calculated on the basis of respective extractive value, and it is equivalent to pharmacopoeial dose which was 20 g of dried leaves.[27]

Based on the screening results, the two potent fractions were identified on the basis of PC. Further, blood samples of these two bioactive fractions were tested for other hematological parameters for antithrombocytopenic activity on the busulfan-induced model, and other biochemical parameters such as total leukocyte count (TLC), hemoglobin count, red blood cells (RBCs), neutrophil count, packed cell volume/hematocrit (PCV/HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC) were also analyzed. The day before the start of the experiment, the PC was analyzed in all the rats. Further, platelets were counted in the experimental and busulfan-treated groups on days 5, 10, 15, and 20 in an automated hematology analyzer (XP-100, Sysmex, Singapore). Blood samples were collected from the retro-orbital vein of an animal by ethylenediaminetetraacetic acid (EDTA)-coated capillary tube and stored in EDTA tube after 1 h of dose administration. After 1 h of dosing, we have collected blood samples on the scheduled time period in order to maintain uniformity. The collected blood samples were subjected to analysis of PC, total leukocyte count, hemoglobin count, RBC, neutrophil count, PCV/HCT, MCV, MCH, and MCHC.[28]

Statistical analysis

All the experiments were performed in triplicate, and data have been expressed as mean with standard error mean. Tukey's test was performed for the evaluation of statistical significance analysis. *P* values were expressed by comparing all datasets with each other at 95% confidence intervals.

RESULTS

Morphological evaluation of *P. guajava* leaves displayed to be simple, green in color, 10–13 cm in length, and 2–6 cm in width. The leaf was opposite and oblong in surface view. The lower surface of the leaf was covered with dense, short, and matted hairs. The transverse section of *P. guajava* leaf showed covered single-layer cuticle in both upper and lower epidermis with six layers of cortex surrounding vascular bundle, and lignified and non-lignified xylem and phloem were present along with arc-shaped vascular bundles [Figure 1a-e]. The pith was made up of large cells. Anomocytic stomata and oil glands were present below the epidermis. An abundant amount of blunt and pointed unicellular trichomes [Figure 1d] were observed. Transverse section of the leaf also showed prismatic and cluster types of calcium oxalate crystals and xylem vessels [Figure 1e]. Powder microscopy showed unicellular trichome, stomata with wavy epidermal cells, and calcium oxalate crystals [Figure 1f-i].

The crude powder of *P. guajava* leaf was green in color having a characteristic odor and astringent taste. Microscopic view clearly showed, prism and cluster type calcium oxalate crystals on the surface of epithelial cells. From a surface view, anomocytic stomata along with fragments of epidermis were embedded [Figure 1g]. Other diagnostic features observed were xylem vessels, calcium oxalate crystals, cork cell, polygonal epidermal cells, and unicellular trichomes having spiral thickening [Figure 1j]. The analyzed parameters of the authenticated plant material were in the acceptable range as mentioned in the WHO guidelines. These diagnostic characteristics of the leaf of *P. guajava* can be used for standardization as well as for of preparation of plant monographs.

Preparation of extract and fractions

One hundred grams of plant material was extracted using hydroalcohol (ethanol 80% v/v) by soxhlation with optimized extraction parameters. The yield was found to be 30.3% w/w. The hydroalcoholic extract was subjected to polarity-based fractionation using *n*-hexane, DCM, *n*-butanol, and remaining aqueous fractions. The yields of fractions were 4.9%, 4.2%, 8.6%, and 11.8% for *n*-hexane, DCM, *n*-butanol, and aqueous fractions, respectively. However, 30 g of hydroalcoholic extract (6% w/w) of the drug was lost during the processing [Figure 2].

TLC fingerprinting of best fractions

n-butanol and aqueous fractions showed the best yield as compared to other fractions. These two fractions (*n*-butanol and aqueous) were further subjected to quality control analysis. The TLC fingerprinting of *n*-butanol and aqueous fractions was developed on TLC plates using toluene:ethyl acetate:formic acid (6:3:1 v/v/v). The number of compounds separated and visualized at a different wavelength is shown in Table 1. The maximum number of compounds observed in the *n*-butanol fraction was 12 and 7 at 254 nm and 450 nm, respectively. However, the aqueous fraction showed the presence of 9 and 6 compounds detected at 254 nm and 450 nm, respectively. Three compounds at R_f 0.03, 0.18, and 0.42 were found to be the common metabolites in both aqueous and *n*-butanol fractions scanned at both 254 and 450 nm, respectively. A similar type of metabolite pattern was observed in aqueous and *n*-butanol fractions irrespective of detection wavelength [Figure 3].

Ultra-performance liquid chromatography–mass spectrometry analysis of extract and fractions

Chromatographic separation of extracts and fractions was carried out by UPLC-MS to identify the number of metabolites present in them and for better interpretation of the diversity of available metabolites. Metabolites present in two best fractions, along with their retention times, experimental *m/z,* and the tentative name of metabolites, were

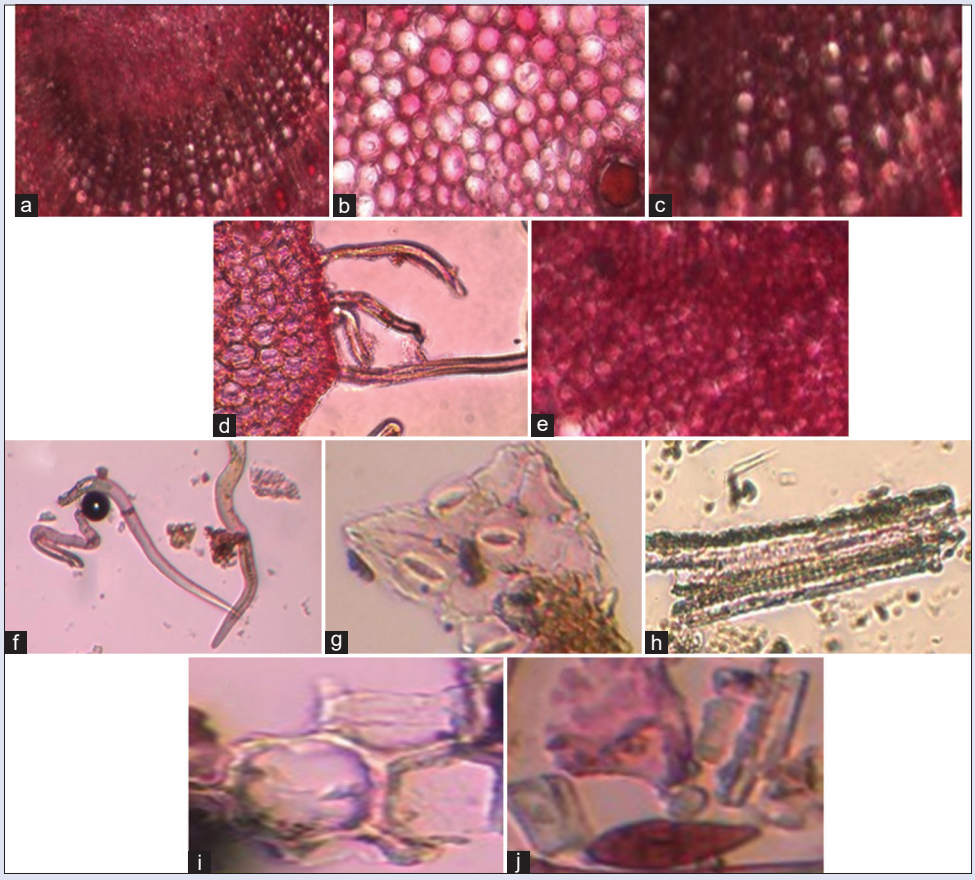

Figure 1: Transverse section of *Psidium guajava* leaves showing (a) sickle-shaped xylems, (b) collenchyma, (c) xylem fibers, (d) trichomes (e) phloem cells, whereas in powder microscopy (f) unicellular trichome, (g) stomata with wavy epidermal cells, (h) xylem vessels and (i) calcium oxalate crystals, and (j) cork cell

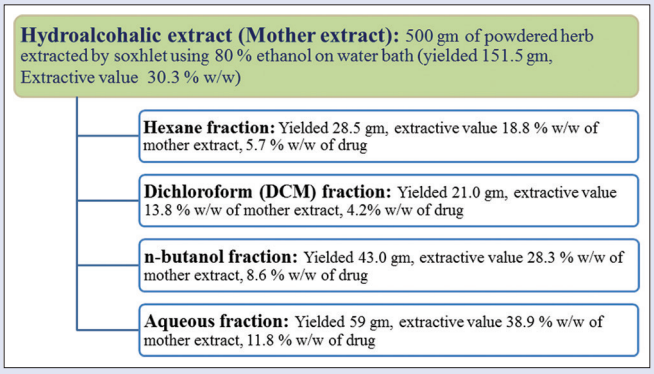

Figure 2: Schematic diagram of extraction, fractionation, and respective yield

summarized in Table 2. Further, these metabolites were tentatively identified by comparing with the experimental mass spectra and data available in the literature. A total of 19 metabolites were identified, of which five metabolites were common and major influencing, present in both aqueous and *n*-butanol fractions [Figure 4]. Five major metabolites (having *m/z* values of 303.05, 302.5, 313.2, 573.51, and 341.32 eluted at 2.80, 3.81, 7.94, 8.55, and 8.69 min, respectively) were found in both aqueous and *n*-butanol fractions. Eleven metabolites eluted at R_t 1.06, 2.80, 3.43, 3.81, 4.2, 4.73, 5.57, 7.77, 7.94, 8.55, and 8.69 min were found only in an aqueous fraction of hydroalcoholic extract, whereas twelve metabolites eluted at R_t 0.78, 2.80, 3.11, 3.81, 4.17, 4.69, 5.55, 5.99, 6.18, 7.94, 8.55, and 8.69 were found only in *n*-butanol fractions. Analyzed metabolites were identified by comparing the mass with the data present in the literature. Identified metabolites were categorized into alkaloids, glycosides, amino acids, flavonoids, lipids, and some carboxylic acids. UPLC-MS study characterized the extract metabolically and identified the compound present on them. These tentatively identified metabolites might be responsible for the therapeutic activity. We have analyzed both lower and major abundant metabolites present in the fractions. The analyzed samples were categorized over a wide polarity range.

The antithrombocytopenic activity of hydroalcoholic extract and its fractions

Initial screening for the identification of the potent active fraction was carried out in rat models separately, and only PC was taken into consideration. Hydroalcoholic extract and all the prepared fractions were treated separately for the busulfan-treated rats. It has been observed that *n*-butanol and aqueous fractions were the best bioactive fractions showing antithrombocytopenic activity as compared to the other fractions. However, the hydroalcoholic extract also showed activity but lesser than other fractions [Figure 5]. Two potent fractions, i.e., *n*-butanol and aqueous fractions, were included for further evaluation of antithrombocytopenic potential.

Platelet count

The developed animal model for thrombocytopenia was confirmed from the PC of the normal and busulfan-treated animals. PC of rats significantly

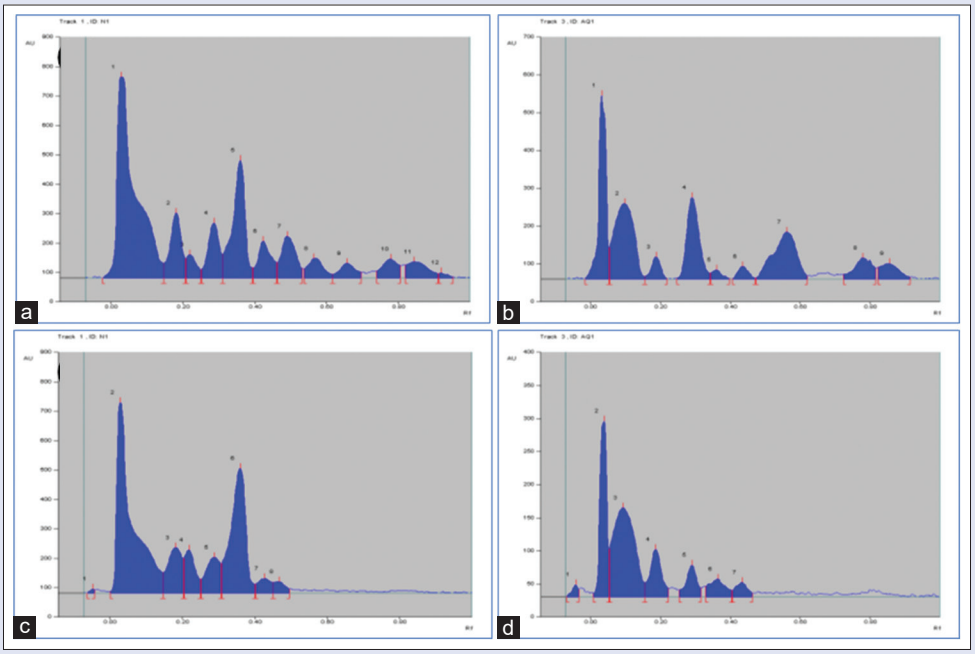

Figure 3: TLC chromatogram of (a) n-butanol, (b) aqueous fractions scanned at 254nm and (c) n-butanol, (d) aqueous fractions scanned at 450nm

Table 1: Thin-layer chromatography fingerprinting profile of aqueous and *n*-butanol fractions of *Psidium guajava* extract scanned at 254 nm and 450 nm

Peak	R_f	254 nm		450 nm	
		n-Butanol	Aqueous	*n*-Butanol	Aqueous
1	0.03	+	+	+	+
2	0.10	–	+	–	+
3	0.18	+	+	+	+
4	0.22	+	–	+	–
5	0.29	+	+	+	+
6	0.36	+	+	+	+
7	0.42	+	+	+	+
8	0.49	+	–	+	–
9	0.56	+	+	–	–
10	0.66	+	–	–	–
11	0.78	+	+	–	–
12	0.84	+	+	–	–
13	0.92	+	–	–	–
Total		12	9	7	6

decreased ($P < 0.05$) after induction with busulfan. Through this model, the PC was successfully reduced, and it was consistently reducing. This rat model was used for the evaluation of the antithrombocytopenic potential of hydroalcoholic extract and fractions of *P. guajava*. The extract caused increased in PC of rats as compared to an untreated rat. However, on days 5–20, the PC decreased significantly ($P < 0.01$) in the toxic group as compared to the normal control group. *P. guajava* hydroalcoholic extract and aqueous fraction in sham control did not show any significant ($P > 0.05$) change in PC with respect to normal control, however, found different symmetrically as toxic control. Further, n-butanol and aqueous fractions of the treatment groups showed significant ($P < 0.05$) reversal with respect to toxic control. This effect is attributable to improved platelet redistribution from the spleen.[29] Among the mentioned two preparations, the aqueous fraction was found to be more significant.

Total leukocyte count and lymphocyte count

Total leukocyte count significantly decreased ($P < 0.05$) in the toxic group (22.9%) as compared to the normal group. The percentage of total leukocyte in the normal and sham control groups (*n*-butanol and aqueous fractions) was found significantly higher ($P < 0.05$) as compared to the treatment group. However, within 20 days of treatment with *n*-butanol and aqueous fractions, leukocyte count was significantly ($P < 0.05$) ameliorated. In thrombocytopenic rats, an aqueous fraction of *P. guajava* showed elevated leukocyte count as compared to hydroalcoholic extract and found significant. *P. guajava* causes no significant change ($P < 0.05$) in the percentage of lymphocyte count against busulfan-treated animals. Even the sham control groups for aqueous and *n*-butanol fractions did not produce any significant change [Table 3].

Hemoglobin content, red blood cell, and neutrophil count

The rats were treated with busulfan, crude extracts of *P. guajava*, and its fractions for 20 days. At the day of 0, 5, 10, 15, and 20, blood was collected from different groups of rats. It has been observed that hemoglobin was significantly decreased ($P < 0.01$) in toxic control as compared to normal control (65.31%). There was no significant difference ($P > 0.05$) observed between the normal control and sham groups. Aqueous and *n*-butanol fractions of *P. guajava* showed a better effect as compared to hydroalcoholic extract and increased the hemoglobin against toxic control [Table 4]. In the case of RBC, little change was observed in the busulfan-treated groups as compared to the normal control group. On the other hand, there were no significant changes found between the normal control and sham groups [Table 4]. Similar results were also observed in case of neutrophil count. No significant changes in the neutrophil count were observed among different groups of rats.

Packed cell volume

At the end of treatment period, HCT level was significantly decreased ($P < 0.01$) in busulfan-treated rats when compared to normal control; contemporarily, there was no significant difference shown between the normal control and sham groups. Busulfan caused decreased on PCV which was reversed in rats treated with aqueous and *n*-butanol fractions of *P. guajava*. Decreased hematocrit level generally causes anemia in humans and in most animals. The present study revealed that aqueous fraction of hydroalcoholic extract was identified as

a bioactive fraction which restores hematocrit level close to the normal range against toxic control [Table 5].

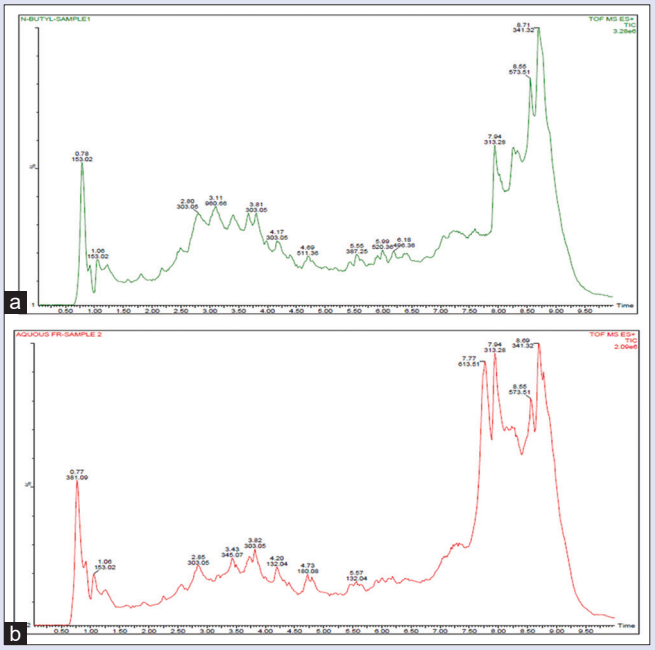

Figure 4: Ultra-performance liquid chromatography-tandem mass spectrometry chromatogram of (a) n--butanol and (b) aqueous fractions

Mean corpuscular volume

MCV is directly related to RBC and increases or decreases in accordance with the average red cell size. This study revealed that MCV significantly decreased ($P < 0.01$) in toxic control as compared to normal control. Our most intriguing findings are revealed that Bioactivity guided fractions from hydroalcoholic extract. Table 5 clearly shows the percentage changes

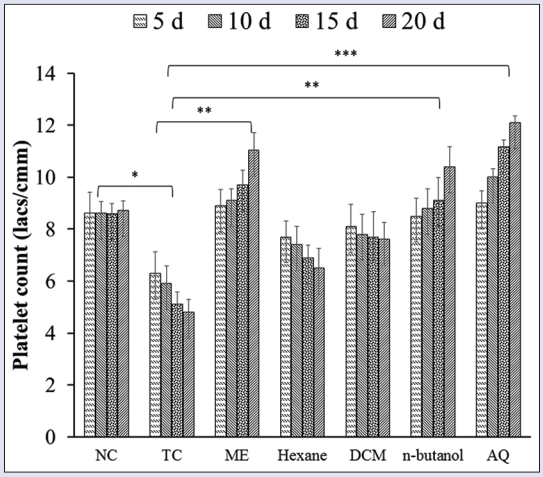

Figure 5: Effect of hydroalcoholic extract and their fraction on platelet count. Data were expressed as mean with relative standard deviation. Significant analysis was performed with a comparison of toxic control for all days except day 5

Table 2: Ultra-performance liquid chromatography-mass spectrometry profiling of aqueous and *n*-butanol fractions of hydroalcoholic extract of *Psidium guajava*

R_t (min)	*m/z*	Metabolite name (mass bank reference)	Category	Aqueous	*n*-Butanol
0.78	153.02	3-hydroxyanthranilic acid (FIO00668)	Amino acid	–	+
1.06	381.09	Cis-Zeatin-9-glucoside (CE000523)	Hormone	+	–
2.80	303.05	Quercetin (CE000168)	Flavonoids	+	+
3.11	960.66	-	-	–	+
3.43	345.07	5-O-Galloylquinic acid	Tannin	+	–
3.81	303.05	Ellagic acid	Flavonoids	+	+
4.17	286.08	Kaempferol	Flavonoid	+	+
4.20	132.04	Methylsuccinic acid (PR100834)	Dicarboxylic acids	+	–
4.69	511.36	Oxysterol	Lipids	–	+
4.73	180.08	(R)-3-Amino-4-phenylbutyric acid (PR100283)	Amino acid	+	–
5.55	387.25	2'-Deoxycytidine 5'-diphosphate (PR100088)	Nucleoside	–	+
5.57	132.04	Methylsuccinic acid (PR100834)	Dicarboxylic acids	+	–
5.99	520.36	Phosphatidylcholine lyso 18:1 (UT001084)	Glycerophospholipids	–	+
6.18	496.36	Palmitoylglycerophosphocholine (MT000099)	Glycerophospholipids	–	+
7.77	613.51	GalNAcFucGlcNAcGA-II (FU000289)	Oligosaccharide	+	–
7.94	313.28	2',3',6-Trimethoxyflavone (BML80200)	Flavonoid	+	+
8.55	573.51	Cycloposine (BSU00004)	Alkaloid	+	+
8.69	341.32	Dicaffeic acid	Phenol	+	+

Table 3: Effect of aqueous and *n*-butanol fractions on total leukocyte and lymphocyte count of busulfan-induced thrombocytopenic rats

Day	Parameters	Normal control	Toxic control	*n*-butanol fraction	Aqueous fraction	Sham *n*-butanol	Sham aqueous
5	Leukocyte (per µL)	9578±18.9**	9050±10.1	8800±12.6**	9750±23.9**	12600±4.4**	11400±3.7**
	Lymphocyte	84.0±0.7*	89.0±0.0	81.0±5.6**	85.0±1.6^{ns}	84.0±2.8*	87.0±4.6^{ns}
10	Leukocyte (per µL)	97350±16.0**	8550±12.4	7550±40.2**	7300±15.4**	11500±3.4**	10500±3.1**
	Lymphocyte	83.5±1.0^{ns}	87.5±2.4	86.0±2.8^{ns}	87.5±0.8^{ns}	81.0±2.7**	85.0±4.7^{ns}
15	Leukocyte (per µL)	95750±18.9**	6450±10.4	7550±8.4**	7650±26.8**	10800±3.1**	12400±3.9**
	Lymphocyte	82.5±1.0^{ns}	82.0±1.7	80.5±0.7^{ns}	86.0±0.0*	88.0±2.9**	80.0±4.1^{ns}
20	Leukocyte (per µL)	94000±12.1**	2200±2.3	9100±20.2**	9800±41.8**	12400±4.3**	11500±3.8**
	Lymphocyte	84.0±1.4^{ns}	88.0±1.6	84.5±2.1^{ns}	87.0±4.8^{ns}	78.0±2.6**	78.0±4.0**

Data were expressed as mean±RSD (n=6). ^{ns}P>0.05, *P<0.05, and **P<0.01. RSD: Relative standard deviation

Table 4: Effect of aqueous and *n*-butanol fractions on hemoglobin, red blood cells, and neutrophil count of busulfan-induced thrombocytopenic rats

Day	Parameters	Normal control	Toxic control	*n*-butanol fraction	Aqueous fraction	Sham *n*-butanol	Sham aqueous
5	RBC (m/mm^3)	7.3±0.1**	6.6±0.1	6.8±0.2^{ns}	6.7±0.2^{ns}	7.9±0.1**	7.21±0.2**
	Hb (g/dL)	14.8±0.3**	12.9±0.5	12.9±0.4^{ns}	13.0±0.1^{ns}	13.5±0.4*	12.4±0.1^{ns}
	Neutrophil (%)	15.5±1.1**	11.0±0.6	18.6±0.5**	15.0±0.1**	13.5±0.4**	12.4±0.1**
10	RBC (m/mm^3)	7.2±0.1^{ns}	6.5±1.1	6.1±0.1^{ns}	6.8±0.8^{ns}	7.8±0.1**	6.79±0.4^{ns}
	Hb (g/dL)	15.3±0.3**	11.8±1.4	11.0±0.6^{ns}	11.2±0.9^{ns}	12.9±0.4^{ns}	11.5±0.6^{ns}
	Neutrophil (%)	15.5±0.3**	12.3±1.4	14.0±0.6**	12.6±0.9^{ns}	12.9±0.4^{ns}	11.5±0.6^{ns}
15	RBC (m/mm^3)	7.8±0.1^{ns}	6.5±1.6	6.2±1.1^{ns}	6.5±0.4^{ns}	8.2±1.1=	7.65±0.2^{ns}
	Hb (g/dL)	15.2±0.2**	11.8±1.1	12.1±0.3^{ns}	12.3±2.2^{ns}	13.7±1.6^{ns}	14.5±1.1**
	Neutrophil (%)	14.5±0.3^{ns}	13.0±1.1	19.0±0.40**	14.6±2.3^{ns}	13.7±1.7^{ns}	14.5±1.1^{ns}
20	RBC (m/mm^3)	7.5±0.2**	5.3±0.4	5.9±1.1^{ns}	6.5±1.2*	7.9±0.1**	7.34±0.2**
	Hb (g/dL)	15.2±0.4**	9.7±0.7	12.2±0.9*	12.9±2.8**	15.7±0.5**	15.0±1.1**
	Neutrophil (%)	14.5±1.1*	12.0±0.8	15.3±0.9**	13.6±2.8^{ns}	15.7±0.5**	15.0±1.1**

Data were expressed as mean±RSD (n=6). ^{ns}P>0.05, *P<0.05, and **P<0.01. RBC: Red blood cells; Hb: Hemoglobin

Table 5: Effect of aqueous and *n*-butanol fractions on mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, and packed cell volume of busulfan-induced thrombocytopenic rats

Day	Parameters	Normal control	Toxic control	*n*-butanol fraction	Aqueous fraction	Sham *n*-butanol	Sham aqueous
5	MCH (pg)	20.0±0.0^{ns}	19.0±1.4	18.5±0.7^{ns}	18.5±0.7^{ns}	20.0±0.7^{ns}	19.5±0.7^{ns}
	MCHC (g/dL)	31.5±0.3^{ns}	32.5±0.7	32.0±0.0^{ns}	31.5±2.1^{ns}	31.5±0.7^{ns}	32.5±0.7^{ns}
	MCV (fL)	63.5±0.3**	58.5±2.1^{ns}	59.0±2.8^{ns}	58.0±2.8^{ns}	61.0±0.0^{ns}**	63.5±1.1**
	PCV (%)	46.0±1.4**	38.5±0.7	39.0±0.0^{ns}	38.5±0.7^{ns}	50.5±2.1**	48.5±0.7**
10	MCH (pg)	23.5±0.3**	18.0±0.0	19.0±1.4^{ns}	18.5±0.7^{ns}	21.0±1.4**	21.0±0.6**
	MCHC (g/dL)	33.0±0.7**	31.0±0.0	31.5±0.7^{ns}	32.0±0.0^{ns}	32.0±1.6^{ns}	31.3±0.8^{ns}
	MCV (fL)	64.5±0.3**	59.5±0.7	61.0±2.8^{ns}	57.0±1.4*	65.0±0.8**	64.5±0.3**
	PCV (%)	46.0±0.7**	37.5±4.9	37.0±1.4^{ns}	38.0±2.8^{ns}	48.0±2.1**	44.0±1.7**
15	MCH (pg)	23.0±0.720**	19.0±1.4	19.0±2.8^{ns}	18.5±2.1^{ns}	19.5±2.1^{ns}	20.0±0.7^{ns}
	MCHC (g/dL)	34.0±0.7**	30.5±0.7	30.5±0.7^{ns}	31.0±2.8^{ns}	30.0±1.0^{ns}	32.4±0.9^{ns}
	MCV (fL)	65.0±0.7^{ns}	61.5±4.9	62.5±7.7^{ns}	59.5±0.7^{ns}	64.5±0.6^{ns}	67.0±0.7^{ns}
	PCV (%)	48.0±0.7**	36.5±2.1	38.5±0.7^{ns}	38.5±3.5^{ns}	47.5±1.8**	44.8±1.7**
20	MCH (pg)	24.5±0.3**	18.0±0.0	21.0±1.4**	18.0±1.4^{ns}	22.0±1.3**	19.0±0.8^{ns}
	MCHC (g/dL)	34.0±1.4**	29.0±1.4	32.0±0.0**	31.5±0.7**	29.5±0.9^{ns}	33.7±0.6**
	MCV (fL)	64.0±0.7^{ns}	63.5±3.5	64.5±4.9^{ns}	57.5±3.5**	64.0±0.7^{ns}	67.0±1.4^{ns}
	PCV (%)	44.0±0.7**	33.5±0.7	38.0±4.2^{ns}	37.5±7.7^{ns}	46.5±1.9**	46.8±1.6**

Data were expressed as mean±RSD (n=6). ^{ns}P>0.05; *P<0.05; and **P<0.01. MCH: Mean corpuscular Hb; MCHC: Mean corpuscular Hb concentration; MCV: Mean corpuscular volume; PCV: Packed cell volume; Hb: Hemoglobin; RSD: Relative standard deviation

in MCV among all the groups. Aqueous and *n*-butanol fractions showed better results as compared to other fractions. Further, this result showed the ameliorative potential of *P. guajava* to normalize the number of MCV.

Mean corpuscular hemoglobin

MCH-depleted and MCH-elevated values were analyzed per groups. No significant changes (P > 0.05) in MCH level were observed in the toxic group as compared to the normal control group. There was no significant change (P > 0.05) observed between the normal control and sham groups. However, *P. guajava* aqueous fraction and hydroalcoholic extract possessed quite similar ameliorative effect against toxic control. Aqueous fraction of *P. guajava* showed the evident effect as compared to hydroalcoholic extract and restored MCH near to normal range [Table 5].

Mean corpuscular hemoglobin concentration

MCHC-depleted and MCHC-elevated values were analyzed in different groups of animals. No significant changes (P > 0.05) in MCHC were observed in busulfan-treated rats as compared to normal control rats. Contemporarily, there were no significant changes found between the normal control and sham groups; hence, it was clear that *P. guajava* does not produce any toxicity. *P. guajava* aqueous and *n*-butanol fractions possessed quite similar ameliorative effect against toxic control and restored MCHC toward the normal range. The results are shown in Table 5.

DISCUSSION

As per the traditional claim, as an ethnomedicine and some recent reports, *P. guajava* was assessed for its antithrombocytopenic activity.[30] Less scientific reports are available for the metabolite responsible for its antithrombocytopenic activity and its mechanism of action. In this context, an attempt was made to produce scientific data regarding its antithrombocytopenic activity and the responsible metabolite through bioactivity-guided fractionations and metabolomic approaches. The busulfan-induced thrombocytopenia in Wistar rats was developed and maintained throughout the study period. The effect of hydroalcoholic extract and its fractions of *P. guajava* was checked on busulfan-induced thrombocytopenic rats. The proposed study revealed that the potentiality of fractions of hydroalcoholic extract (*n*-butanol and aqueous fractions) of *P. guajava* is more active as a platelet-augmenting agent as compared to other fractions as well as hydroalcoholic extract.

Remarkably, both aqueous and *n*-butanol fractions of hydroalcoholic extract of *P. guajava* showed a significant ameliorative potential (P < 0.05) against toxic control and reversed the hematological parameters such as platelet, leukocyte, total lymphocyte, neutrophil count, hemoglobin, RBC, MCV, PCV/HCT, MCH, and MCHC. There was no significant difference observed in normal animals treated with *n*-butanol and aqueous fractions of *P. guajava* (sham control groups). The reversal potential of the aforementioned hematological parameters was found to be more

in an aqueous fraction as compared to other fractions and even hydroalcoholic extract of *P. guajava*.

The data obtained from the present study revealed that there could be some bioactive compounds present in *P. guajava* that can enhance hematological parameters in thrombocytopenic rats. Bioactive compounds, present in leaves of *P. guajava,* prevented platelet destruction in the blood and ultimately enhance platelet life in circulation. Based on the present finding, it can be considered that *P. guajava* may be a promising platelet-increasing agent or antithrombocytopenic agent. Previously, *P. guajava* leaf extract has been tested *in vitro* and also in human found inhibitory effect against dengue virus.[31] In a healthy volunteer, juices did not cause any significant alteration of the platelet aggregation.[32]

Thrombocytopenia is another clinical manifestation and is common in patients with either mild or severe cases of dengue virus infections. The PC drops below normal level (150,000–450,000 platelets/μL) and may reach as low as <40,000 platelets/μL of the blood sample. Other symptoms include a tendency to bleed excessively into mucous membranes, especially during menstruation.[33] Both aqueous and alcoholic extracts of *P. guajava* leaves showed activity against viral infection, and these extracts were able to reverse the blood count affected by infection.[34] Earlier, *P. guajava* at 250 mg/kg increased PC, and it was decreased at a dose of 500 mg/kg. However, the hematological parameters were balanced by both doses as compared to normal control.[19] However, in our study, no significant changes in hematological parameters were observed in the sham control groups. This result signifies the safety of extract at a used dose equivalent to pharmacopoeial dose.

The previous finding suggested that guava juice and extract increased the platelet number and megakaryocyte (MK), respectively, in mice, and the major metabolite responsible for antithrombocytopenic activity is trombinol.[20] It may be present in hydroalcoholic extract, but we did not found in our extract; it may be due to geographical and species variations. We evaluated the effect of *P. guajava* hydroalcoholic extract and its fractions against busulfan-induced rat model and found significant ($P < 0.05$) ameliorative effect in PC. UPLC-MS analysis revealed the presence of major phenolic and flavonoid compounds such as quercetin, ellagic acid, and kaempferol. Quercetin decreased the adenosine diphosphate-induced platelet aggregation.[35] Ellagic acid caused increased in platelet production by overexpression of cyclooxygenase pathway.[36] Platelet production is a two-step process consisting of megakaryopoiesis and thrombopoiesis.[37] Megakaryopoiesis is a complex process that involves the differentiation of hematopoietic stem cells (HSCs) to platelet production when the cytoplasm of MKs extends and to form a prothrombocyte.[38] Natural compounds having antioxidant properties, especially flavonoids, are able to differentiate the HSCs which helps to increases platelet production.[39] Chromatographic analysis revealed the enrichment of flavonoid compounds in bioactive as well as in crude extract. From our screening study and pevious findings revealed that hydroalcoholic extract of P. *guajava* potentially enhances level of PC. However, in our study, we have found that bioactive fraction showed better activity as compared to hydroalcoholic extract, and it may be due to the presence of oxysterol which does not have any significant adverse effect of platelet aggregation.[40]

P. guajava has been widely used as a folk medicine to increase thrombocyte count in dengue viral infection.[41] Cytokine physiologically regulates the production of thrombocyte. Deficient in cytokine level significantly reduces the platelet production and leads to the thrombocytopenic condition. Some bioactive compounds stimulate the production of cytokine, and this molecular explanation of thrombopoietin justifies the ancient medicinal theory for the functional usage of *P. guajava* in thrombocytopenia-dengue fever.[42] Few studies have been reported about the potential of phenolic compounds such as gallic acid, ellagic acid, quercetin, and kaempferol against dengue virus.[6,43] Quercetin-like bioflavonoid showed the antiviral activity against dengue type 2 virus.[44]

An analytical study is one of the best approaches to monitor and evaluate the quality of the ethnomedicine as well as raw materials because this method has the ability to explore qualitative and quantitative aspects of all bioactive metabolites present in herb and its extract. TLC is one of the prominently used analytical techniques for fingerprint analysis and to evaluate the quality control parameters of medicinal plants and their related products. Simultaneously, results obtained from this method could be directly evaluated by visual inspection.[45] This technique was mainly used for identification and authentication of medicinal plants as well as used to separate different constituents to provide useful qualitative and quantitative data that are reliable, accurate, and economic for various applications including quality control and standardization of food and marketed herbal formulations.[46] In the present study, TLC method was developed for fingerprint analysis to identify and authenticate the different fractions of *P. guajava* leaves. In order to get the reproducibility of pharmacodynamic properties of herbal extract, TLC fingerprinting is one of the major qualitative tools of analysis. In our study, we have analyzed the bioactive fraction through TLC, and metabolites were identified through liquid chromatography–mass spectroscopy. Although UPLC-MS analysis, we have identified and categorized the metabolites present in it. The *P. guajava* leaves possess a rich amount of tannins, phenolics, flavonoids, sesquiterpene, essential oils, triterpenoid acids, and many more. These secondary metabolites present in *P. guajava* help in health restoration. The ethanolic extract of *P. guajava* exhibited a variety of bioactive compounds, which is responsible for its numerous medicinal effects. Many phytochemical screening of leaves of *P. guajava* and its by-products reported the presence of different categories of metabolites such as anthocyanins, carotenoids, essential oils, fatty acids, lectins, phenols, saponins, tannins, triterpenes, and Vitamin C.[47] The availability of numerous bioactive compound supports the claims made by the traditional healers about *P. guajava.* Based on the finding, the present study suggests that *P. guajava* passes excellent antithrombocytopenic activity rat model.

CONCLUSION

The metabolically characterized hydroalcoholic extract of *P. guajava* and its bioactive fractions ameliorates PC in busulfan-induced thrombocytopenic rats. It is the first scientific reports on the bioactivity-guided fractionations and identification of best bioactive fraction, which can be explored as phytopharmaceutical for the management of platelet-related disorders. However, this is a preliminary study, and more work is needed to isolate and identify the biologically active ingredients of *P. guajava* that is responsible for antithrombocytopenic activity.

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Conflicts of interest

There are no conflicts of interest.

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