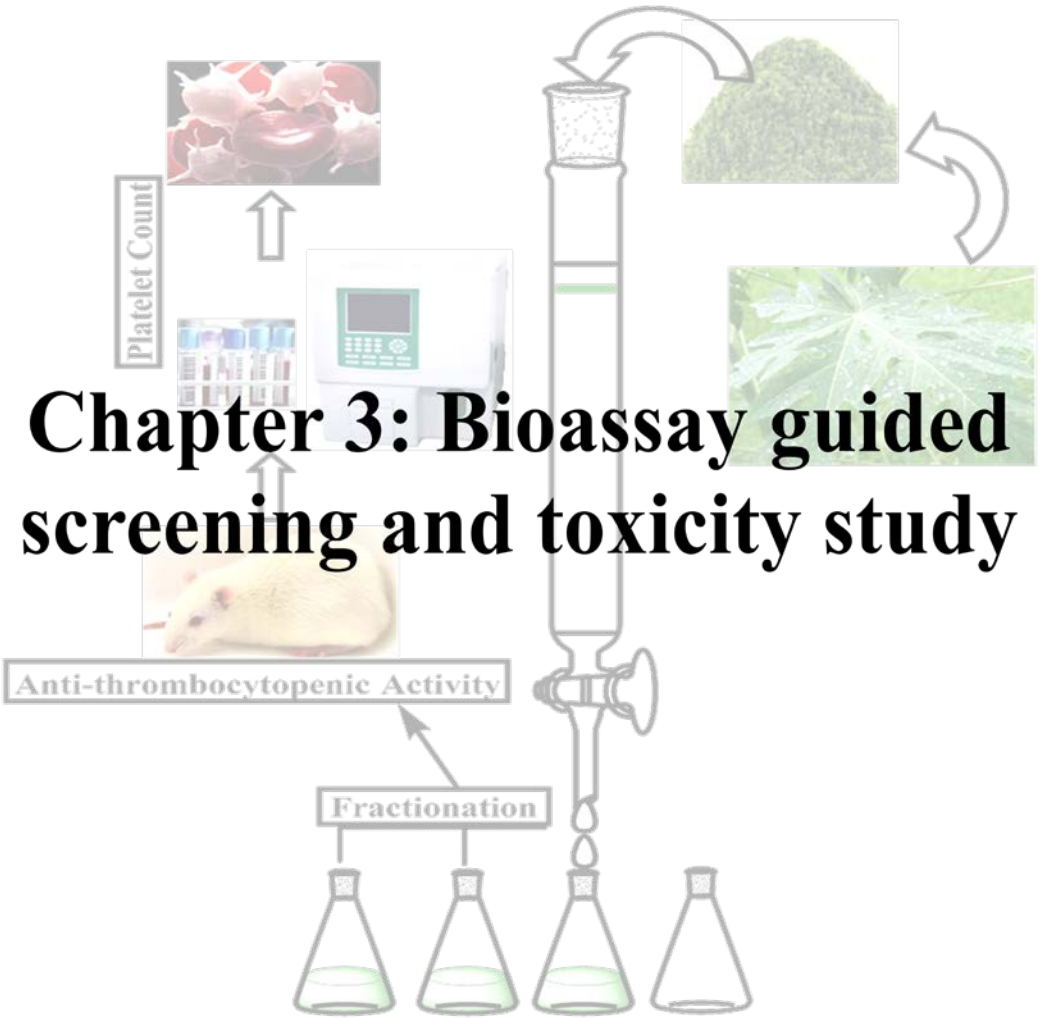


Chapter 3: Bioassay guided screening and toxicity study

3: Bioassay guided screening and toxicity study

3.1 Introduction

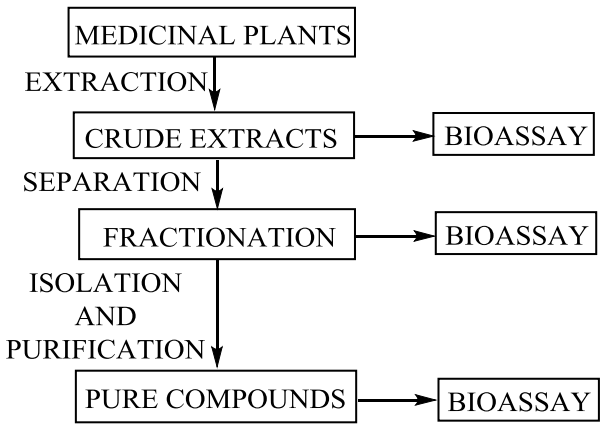
In the early years of evolution of medicinal science, the drug discovery must have been made by sharp observations or occurred by sheer serendipity. In the last two decades, with better understanding of the disease at molecular levels, new drugs are designed with appropriate structure for exclusive action. On the basis of target receptors, a large number of compounds are screened for the desired binding or modification of phenotypes of biological systems [1]–[4]. The choice of the compound is simplified by a 'rule of 5', an algorithm, based on pharmacokinetic properties of human body like absorption, distribution, metabolism and excretion (ADME) given by Christopher Lipinski to predict oral drug ability of a compound [5], [6]. The automated screening, known as High Throughput Screening (HTS) may be experimental or virtual, was expected to generate many new drug molecules to fight diseases [7]. However against this expectation, even after spending a good fortune on HTS by pharmaceutical companies, only few new molecules were given the status of drug by FDA [8]–[11].

There are many reasons for the failure of the HTS technique for discovery of drugs, like *in vivo* behavior of the chemical may be different from the *in vitro* model used for the screening [12]. Many effective drugs exhibit polypharmacology *i.e.* acting on more than one target or effective treatment of disease may require modulation of more than one mode in parallel for modification of phenotypes of biological systems. 'Network Pharmacology' created to extract information about drugs that commonly act on multiple targets and drug that can act on multiple diseases by integrating polypharmacological database with databank of human disease gene, has been proposed as an alternate approach for drug discovery [13], [14].

Natural products with stupendous molecular biodiversity provide an enormous source of compounds that can be used as drugs, and in fact one-third of the FDA approved drugs been derived from the natural products [15].

The drug development from natural product is, apart from being complex, time and resource consuming. It requires collaboration between botanist/zoologist, chemist, pharmacologist and a clinical expert [16], [17]. Many pharmaceuticals companies have eliminated or scaled down their natural product research due to long and tedious process in the drug discovery [18]–[20].

The drug discovery from medicinal plant is initiated by selection of plant/s based on ethnobotanical and taxonomical principles. The next step is to check bioactivity of the whole plant or a part of the plant on the basis of ethnopharmacological data. According to Choudhary et al, on the basis of target, the biological assay can be broadly divided into various groups as a)assaying effect of drug on isolated subcellular systems like proteins, DNA etc. b) using cell lines and tissues c) using lower organisms as targets and d) use of animal model as target [21]. Depending upon the nature of bioactivity to be studied, the suitable target is used. Once the target is decided, the crude extract of the selected plant material is screened for its pharmacological efficacy and toxicity. The crude extract is fractioned, each fraction is subjected to selective bioassay, and the fraction exhibiting better activity is fractioned again. The process of fractionation and sub-fractionation is repeated till a pure compound or a lead compound is isolated.



Scheme 3.1: General scheme of bio-guided assay

In the present work, due to lack of nucleus, platelets are very difficult to study *in vitro*, hence animal model was found to be the most suitable as target to study

bioactivity of the *Carica papaya* leaves by monitoring number of platelets. The bio-guided fractionation study was carried out in two parts.

The first part of the study (Part A) was carried out on healthy Sprague Dawley Rats (SD rats) as target.

a) Primary bioassay of crude water extract of *Carica papaya* leaves and toxicity study.

b) Screening of fractions separated by chromatographically on the basis of polarity.

c) Screening of the fraction separated on the basis of phytochemical functional groups.

The second part of the bioassay experiment (Part B) is about preparation and use of thrombocytopenic animal model for identification of a lead compound.

3.2 PART A

3.2.1 Materials and methods

3.2.1.1 Collection and authentication of plant material

Collection and authentication of plant material were done as described in section 2.2.1.1.

3.2.1.2 Chemicals

All chemicals used were of analytical reagent grade (AR). Acetic acid, ethanol, chloroform, cyclohexane, petroleum ether, liquor ammonia, ethyl acetate, n-hexane, acetone, methanol (HPLC grade) were purchased from the Merck (India).

Conductivity water was used wherever required. Agar was obtained from HiMedia Lab (India).

3.2.1.3 Animal Husbandry

The test system used in this study was on both male and female Sprague Dawley Rats (SD rats) aged between six to seven weeks, weighing about 90 to 100 g. The individual body weights were within ±20% of the mean of each sex.

Food (commercially available rat chow, standard laboratory diet: M/s Pranav Agro Ltd Baroda, India) and water was provided *ad libitum*. Experiments were performed in accordance with guidelines of Institutional Animal Ethical Committee (Approval no CPCSEA.827/ac/04), a Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

All experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee prior to initiation of the experiment.

The animals were housed in polypropylene cages (three animals per cage) and placed in the experimental room where they were allowed to acclimatize for a week before experiment.

A 10% air exhaust-conditioning unit was maintained along with a relative humidity of 60 ± 5% and a temperature of 25 ± 3 $^{\circ}$C in the animal house facility. A 12:12 light: dark cycle was also regulated for the experimental animals.

3.2.1.4 Biochemical analysis

Blood samples were collected on 1st and 7th day by retro-orbital puncture under diethyl ether anesthesia from the rats. The samples were transferred into K_3EDTA vacunities for CBC count using midray 2800E automated haematoanalyser.

3.2.2 Sub Chronic Study

3.2.2.1 Experimental Design

The sub chronic toxicity study was carried out according to the OECD Guidelines No. 408 for the "Repeated Dose 90-day Oral Toxicity Study in Rodents" with some modifications, namely the temperature and humidity of the room [28].

The rats were divided randomly into two groups, one control group and one test group each consisted of six rats, three male and three female weighing about 90 to 100 g.

3.2.2.2 Toxicity study

All the test preparations were administered as a suspension in 0.2% agar, orally. The test group was given the *C. papaya* extract at 1 g/kg BW delivered in a 2 mL volume using intubation needle given daily at about the same time for 90 days. Control rats received the same volume of normal saline water as the amount given to the test groups.

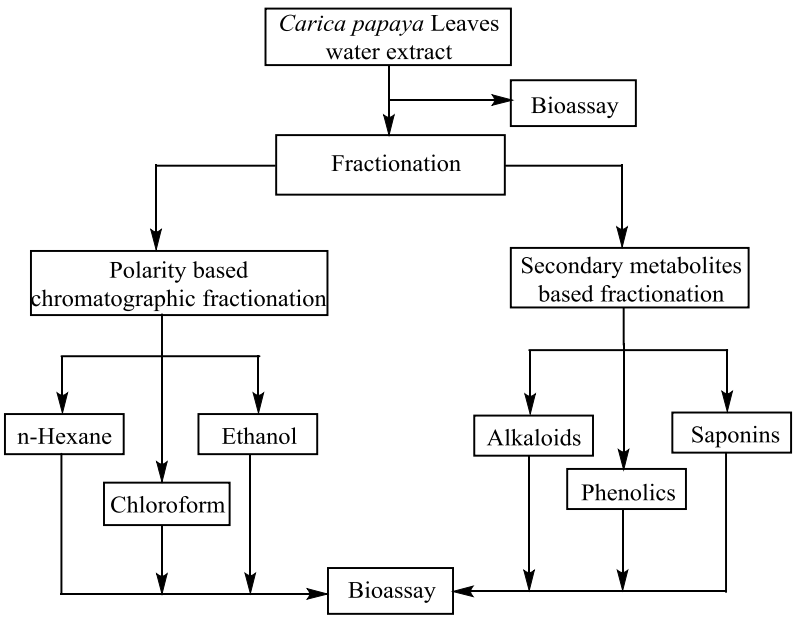
Individual rats were weighed before the commencement of the experiment and then weighed once on the tenth day. Hematological parameter studies were performed after every 10^{th} day of the dosing. The food and water were measured weekly and the differences were calculated and regarded as food and water consumption (g/rat/week). Clinical observations were made once a day for mortality, moribund and ill health or reaction to treatment, such as changes on skin and fur, eyes and mucus membrane, behavior pattern, tremors, salivation, diarrhea, sleep and coma.

The results of sub chronic toxicity studies are given in Table 3.1.

Table 3.1: Result of Sub chronic toxicity

Days of Treatment	Platelet Count Mean±SD (10^9/L)
Control	599.17±81.65
10th day	579.17±63.14
20th day	341.33±34.26
30th day	511.17±93.30
40th day	540.67±115.40
50th day	603.00±41.65
60th day	632.00±94.10
70th day	655.17±25.77
80th day	680.50±46.15
90th day	804.33±77.10

This part of the work involved use of healthy, normal rats as target for bioassay. Followed primary screening of *Carica papaya* leaf extract, bioassay guided fractionation experiments were designed as per following Scheme 3.2.



Scheme 3.2: Bioassay guided fractionation experiments

3.2.3 Primary screening of crude water extract of *Carica papaya* leaves

The rats were divided randomly into four groups, one control group and three test groups each consisted of six rats, three male and three female weighing about 90 to 100g. All the test preparations were administered as a suspension in 0.2% agar, orally. The test group was given the *C. papaya* extract at 10 mg/kg, 100 mg/kg, and 1000 mg/kg BW delivered in a 2 mL volume using intubation needle daily at about the same time for 7 days. Control rats received the same volume of normal saline water as the amount given to the test groups.

The results of bioactivity of crude water extract of papaya leaves are summarized in Table 3.2.

Table 3.2: Result of primary screening of crude water extract of *Carica papaya* leaves

	Platelet Count Mean±SD (10^9/L)
Control	497.00±32.67
10 mg	779.17±51.54
100 mg	754.17±81.98
1000 mg	774.00±165.57

3.2.4 Screening of the fractions separated on the basis of polarity

3.2.4.1 Chromatographic fractionation

A total of 1 kg of dried grounded powder of *Carica papaya* leaves was extracted by percolation method with 1000 mL of water. The aqueous solution was evaporated to dryness under vacuum. The dried water extract was adsorbed on silica gel (60-120 mesh) and poured over silica packed column. The different fractions were collected using n-hexane, followed by chloroform, and finally ethanol 1000L each as eluting solvents. Solvent distillation and drying of each fraction was done under vacuum. Concurrently each fraction was subjected to bioassay on Sprague Dawley rats.

3.2.4.2 Bioassay

The rats were divided randomly into five groups, one control group and four test groups each consisted of six rats, three male and three female weighing about 90 to 100 g. All the test preparations were administered as a suspension in 0.2% agar, orally. Each test group was given 200 mg/kg BW of the different extracts delivered in a 2 mL volume using intubation needle daily at about the same time for 7 days. Control rats received the same volume of normal saline water as the amount given to the test groups.

The results in Table 3.3 comprises of bioassay screening of fraction of different polarities.

Table 3.3: Results of screening of fractions on different polarities

Extracts	**Platelet Count Mean±SD (10^9/L)**	
	1st day	**7th day**
Control	548.33±18.77	663.66±26.76
Hexane	566.33±55.95	616.33±38.00
Chloroform	537.67±89.37	639.00±11.79
Ethanol	600.33±20.50	698.67±58.71
Water	500.33±128.71	700.33±93.22

3.2.5 Screening of fractions of different phytochemical functional groups

3.2.5.1 Separation of major phytochemical groups

Preliminary phytochemical screening and survey of literature revealed the presence of primary metabolites like carbohydrates, proteins, amino acids and secondary metabolites like iridoids, flavonoids, phenolics, alkaloids and saponin glycosides in *C. papaya* leaves [22]. The three major groups of phytochemicals namely alkaloids [23], phenolics [24] and saponins were extracted from water extract by conventional methods.

3.2.5.1.1 Extraction of Alkaloids

The dried, powdered leaves (500 g) of *Carica papaya* were refluxed for 12 h in ethanol and acetic acid (90:1). The extracted was filtered and again the same process was repeated twice. The combined alcoholic extracts were concentrated to a small volume at ordinary pressure initially and later under reduced pressure. The residue was treated with water (500 mL), and acidified with acetic acid and kept overnight. Celite filtration was done to remove sticky resinous material from aqueous extract. The residue was again treated with dilute acetic acid till it was free from alkaloidal material. The combined aqueous extracts were concentrated under *vacuum*. The clear aqueous solution was repeatedly extracted with ether for removal of chlorophyll and highly non-polar phytochemicals. The aqueous fraction was again treated with acetone for separation of highly polar compounds and the resultant solution was centrifuged, decanted, distilled and concentrated under *vacuum.* The concentrated portion was diluted with 500 mL distilled water. The aqueous solution was basified using liquor ammonia and kept overnight. The liberated base was thoroughly extracted with diethyl ether, dried over Na_2SO_4 bed, and concentrated. Crude alkaloids were obtained which appeared as sticky light brown solid [23].

The extracted alkaloidal group was confirmed using dragendorff reagent which showed brown color spot on TLC after derivatization.

Figure 3.1: TLC run with EtOAc: MeOH: HCOOH (5:1:1) solvent and derivatized with dragendorff reagent

3.2.5.1.2 Extraction of Phenolics

The air-dried and finely powdered parts of *Carica papaya* (280 g) were macerated overnight, in petroleum ether: cyclohexane (50%) for defatting at room temperature. The residue was extracted with methanol, distilled and the remaining residue was further hydrolyzed with 4 M hydrochloric acid. The hydrolyzed fraction was filtered and subjected to sequential extraction with ethyl acetate three times [24]. The ethyl acetate fraction was concentrated and dried and subjected to confirmatory test. The presence of phenolics was confirmed by a) Formation of dark green color on addition of $FeCl_3$ and b) Formation of bulky white precipitate in the lead acetate test.

3.2.5.1.3 Extraction of Saponins glycoside

The dried, powdered leaves (1000 g) of *Carica papaya* were refluxed with 70% aqueous EtOH at the boiling point for 2 h for three times. The extract was concentrated under reduced pressure and then partitioned between n-BuOH and H_2O. The n-BuOH extract was subjected to Diaion HP-20 column chromatography and eluted with gradients of H_2O and 20%, 70% and 95% EtOH, successively [25], [26].
The eluted fraction with 70% EtOH were concentrated and analysed by TLC on silica gel plates (Merck, Darmstadt, Germany) developed in ethyl acetate: formic acid: methanol (5:1:1 v/v/v) and visualized by spraying the TLC plates with anisaldehyde sulfuric acid reagent followed by heating at 130 $^{\circ}$C [27]. Violet, blue, red, grey or green spot on TLC indicated the presence of saponin in the crude extract.

Figure 3.2: TLC run with EtOAc: MeOH: HCOOH (5:1:1) solvent and derivatized with anisaldehyde sulfuric acid reagent followed by heating at 130°C.

3.2.5.2 Bioassay

The rats were divided randomly into four groups, one control group and three test groups each consisted of six rats, three male and three female weighing about 90 to 100g. All the test preparations were administered as a suspension in 0.2% agar, orally. The test groups were given 200 mg of various phytochemical fractions of *C. papaya leaf extract* (PLE) per kilogram body weight of each rat, in a 2 mL volume using intubation needle daily at about the same time for 7 days. Control rats received the same volume of normal saline water as the amount given to the test groups.

The bioactivity of different fractions separated on the basis of phytochemical groups separation results are summarized in Table 3.4.

Table 3.4: Bioassay using different phytochemical functional group

Extracts	**Platelet Count Mean±SD (10^9/L)**	
	1st day	**7th day**
Control	573.33±48.23	622.33±28.71
Water	663.33±20.26	791.67±13.65
Saponins	515.33±31.26	586.00±37.64
Phenolics	509.67±13.20	543.00±3.61
Alkaloids	611.67±52.70	1004.00±53.51

3.3 Discussion

The results of the primary screening of the *Carica papaya* leaf extract indicated increase in platelet counts by about 40% irrespective of the dose [Table 3.1]. These results are similar to the earlier reports [29].

The results of bioassay of *Carica papaya* leaf fractions with different polarities suggested the pharmaceutically active compound to be more polar as the fractions extracted with lower polarity solvent system did not show any significant rise in the platelet counts [Table 3.2].

The results of screening of different phytochemical groups [Table 3.3] indicated no conclusive rise in platelet counts when the targets were administered with phenolics and saponin groups. A substantial rise in platelets was observed from 611.67 ± 52.70 10^9/L to 1004.00 ± 53.51 10^9/L, when the targets were given alkaloids. These results pointed out that the alkaloids present in *Carica papaya* leaf are probably responsible for platelet boost.

The results of the toxicity studies [90 day protocol Table 3.4] revealed the *Carica papaya* leaf to be safe for oral administration, these results augmented the previous observations made by Tarkang et al, for toxicity studied made with 28 days and 90 days protocol [30].

3.4 PART B

3.5 Introduction

As mentioned previously in the introduction the bioactive studies in the section were carried out on a thrombocytopenic model. The blood platelets being a non-nucleated, the *in vitro* study is not possible, so, scientists have developed thrombocytopenic models of different animals for *in vivo* studies. Review of literature on induction of thrombocytopenia in different animals is summarized in Table 3.5.

Table 3.5: Different types of model preparation of thrombocytopenia

Sl. No	Species	Thrombocytopenic agent (Dosage) & mode of insertion
Rabbits	Female mixed-breed	Arachidonic acid (1 mg/Kg BW) intravenously [31]–[33]
		Collagen (30 μg/Kg BW) intravenously [33]–[36]
	Male albino	Busulfan (50 mg/Kg BW) subcutaneously [37]–[39]
Rodents	Male Swiss-Webster Mice	Collagen (15 μg/Kg BW) + epinephrine (1.8 μg/Kg BW) intravenously [33], [40], [41]
	BALB/c Mice	Cyclophosphamide (200 mg/Kg BW) intravenously [42], [43]
	Female BALB/c Mice	Monoclonal PLT antibody (10^8 platelets) intraperitoneally [44]–[46]
	(CAST/Ei) Mice	scFv4-20 (10 mg/g BW) intravenously [43], [47], [48]
	Hamsters	Collagen (50 μg/kg BW) + adrenaline (10 μg/Kg BW) intravenously [49]
	Guinea pigs	Platelet Activating Factor (0.03–0.04 μg/Kg BW) intravenously [50], [51]
		Thrombin (60 U/Kg BW) intravenously [31], [49], [52]
	Male Sprague-Dawley Rats	Cyclophosphamide (400 mg/Kg BW) intra-peritoneally [53]–[55]
Sheep	Male	Busulfan (900 mg/m^2 body surface area) subcutaneously [56]–[58]

In the present study, after trying various reagents, busulfan was found to be the best chemical for the induction of sustainable thrombocytopenia in Wistar rats.

3.5.1 Materials and method

3.5.1.1 Chemicals and reagents

All analytical grade solvents and acids (methanol, n-butanol, glacial acetic acid, hydrochloric acid, sulfuric acid, chloroform, ethyl acetate, petroleum ether and ammonia) were purchased from Fisher Scientific Pvt. Ltd. (India). Busulfan was purchased from Sigma Aldrich (India). Polyethylene glycol 400 was purchased from Merck (Darmstadt, Germany). Agar was obtained from HiMedia Lab (India).

3.5.1.2 Animal husbandry

Wistar rats, both male and female, weighing 200–250 g were obtained from the animal house of B. V. Patel PERD Centre, Ahmadabad. Animal housing and handling were performed in accordance with Good Laboratory Practice (GLP) mentioned in CPCSEA guidelines. The animal house is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, vide registration no. 1661/PO/a/12/CPCSEA, dated 21 November 2012 [59]. All experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee prior to initiation of the experiment. The animals were housed in polypropylene cages (three animals per cage) and placed in the experimental room where they were allowed to acclimatize for a week before experiment. A 10% air exhaust-conditioning unit was maintained along with a relative humidity of 60 ± 5% and a temperature of 25 ± 3 °C in the animal house facility. A 12:12 light:dark cycle was also regulated for the experimental animals. Amrut certified rodent diet (Maharashtra Chakan Oil Mill Ltd) and tap water (boiling hot water cooled to room temperature) were provided *ad libitum* to the experimental animals.

3.5.2 Animal model development

Animals were randomly distributed in 2 groups each consisted of 3 male and 3 female (n=6) *viz*. normal control and disease control. The optimization and titration of busulfan concentration was done as reported by Kitchens et al on Wistar rats by subcutaneously injecting busulfan in PEG 400 [60]. 5 mg busulfan/kg body weight was found to the optimum dose for making sustainable working model of Wistar rat. Thrombocytopenia was induced by subcutaneous injection of busulfan at a dose of 5 mg/kg in Wistar rats on day 1, 5, 10 and 15. Platelet counts were determined on day 1 prior to the initiation of dosing and on day 20 in an automated hematology analyzer, VetScan HM-5 (Abaxis Inc., Union City, CA, USA).

The results of dose optimization for model preparation in wistar rats are summarized in Table 3.6.

Table 3.6: Optimization of doses for model preparation

Animal dosage	**Platelet Count Mean±SD (10^9/L)**			
	Day 1st		**Day 14th**	
	Control	**Test**	**Control**	**Test**
25 mg/kg BW	619.83±98.06	801.33±65.29	654.00±68.66	0.50±1.22
10 mg/kg BW	837.55±130.23	692.83±56.02	873.75±41.17	22.17±21.01
5 mg/kg BW	800.99±149.92	769.83±50.12	861.85±91.02	77.50±31.53

3.5.3 Bioassay of major phytochemical groups on thrombocytopenic model

3.5.3.1 Bioassay

The major phytochemical groups namely phenolics, alkaloids and saponins along with water extract of *Carica papaya* leaves were screened for antithrombocytopenic activity using Wistar rat model as target. For bioactivity studies, animals were randomly distributed in 6 groups (n=6) *viz*. normal control, disease control and 4 test groups for screening the activity of different phytochemicals. Thrombocytopenia was induced by subcutaneous injection of busulfan in 10% PEG at a dose of 5 mg/kg in

Wistar rats on day 1, 5, 10 and 15. The animals were treated with the test preparation for a period of 20 days which started on the same day as that of the busulfan dosing. All the test preparations were administered as a suspension in 0.2% agar, orally. Platelet counts were determined on day 1 prior to the initiation of dosing of either test preparation or busulfan and on day 20 in an automated hematology analyzer, VetScan HM-5 (Abaxis Inc., Union City, CA, USA).

The results of screening of various phytochemical groups for bioactivity are summarized in Table 3.7.

Table 3.7: Bioassay of different phytochemical groups on thrombocytopenic model

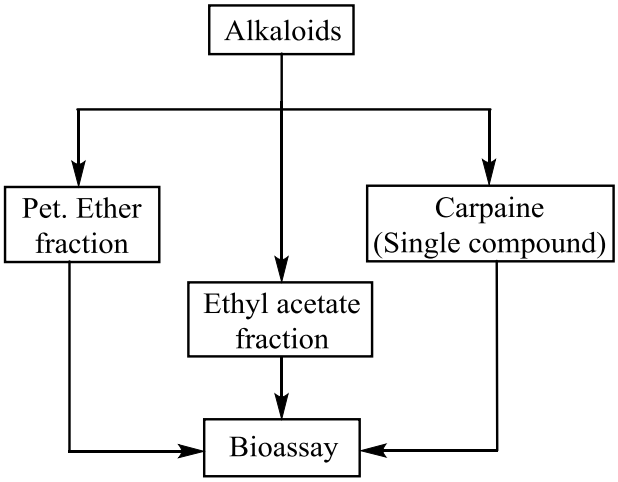
Extracts	**Platelet Count Mean±SD (10^9/L)**	
	1st day	**20th day**
Control	623.25±105.32	704.75±132.92
Diseased	800.75±142.57	78.75±27.28
Water	782.50±189.19	33.50±15.24
Saponins	883.75±133.30	112.50±24.12
Phenolics	813.50±153.21	72.25±17.69
Alkaloids	973.00±243.96	313.25±50.74

3.5.4 Sub-fractionation and bioactivity of alkaloidal fraction

The results on bioassay of various phytochemical groups indicated alkaloidal fraction to be most effective in controlling the drop of platelet counts compared to the untreated and treated with other fractions. The alkaloidal fraction was further sub fractioned for bioassay and identification of a lead compound.

There are many alkaloids present in *Carica papaya* leaf like carpaine, pseudocarpaine, dehydrocarpaine I and II, choline, and methyl derivative of carpaine. Among them carpaine is the major alkaloid component [23].

For bioassay studies, the alkaloid fraction was sub-fractionated as per following Scheme 3.3.



Scheme 3.3: Sub-fractionation of alkaloidal group

3.5.4.1 Bioactivity of sub-fractions of alkaloid group

Each subfraction of alkaloidal fraction was screened for the antithrombocytopenic activity using thrombocytopenic model as target.

The animals were randomly distributed in 5 groups (n=6) *viz*. normal control, disease control and 3 test groups for screening the activity of different. Thrombocytopenia was induced by subcutaneous injection of busulfan in PEG 400 at a dose of 5 mg/kg in Wistar rats on day 1, 5, 10 and 15. The animals were treated with the test preparation for a period of 20 days which started on the same day as that of the busulfan dosing. All the test preparations were administered as a suspension in 0.2% agar, orally. Platelet counts were determined on day 1 prior to the initiation of dosing of either test preparation or busulfan and on day 20 in an automated hematology analyzer, VetScan HM-5 (Abaxis Inc., Union City, CA, USA).

The dose of all the extracts was 200 mg/kg whereas that of carpaine was 2 mg/kg based on its extractive value. The results are summarized in Table 3.8.

Table 3.8: Bioassay of sub-fractions of alkaloidal group on thrombocytopenic model

Extracts	Platelet Count Mean±SD (10^9/L)	
	1st day	20th day
Control	748.50±203.96	718.75±157.89
Diseased	730.25±135.53	81.50±30.21
Pet. Ether fraction of alkaloids	660.50±99.47	759.75±181.44
Ethyl acetate fraction of alkaloids	578.25±54.79	632.25±116.23
Carpaine (Single compound)	623.00±168.74	555.50±42.99

3.6 Discussion

The results [Table 3.6] confirmed successful making of a thrombocytopenic model using busulfan at the optimized does.

The results of screening of phytochemical groups for bioactivity on thrombocytopenic model indicated that alkaloids were most effective in controlling the drop of platelet. The bioassay of sub fractions further confirmed that the compound responsible for the anti-thrombocytopenic activity belongs to alkaloid group and it may be carpaine, the chief alkaloid present in *Carica papaya* leaf.

3.7 Quantitation of carpaine in different extracts by LCMS-MS

As the alkaloidal fraction was found to be more effective, attempts were made to develop a method for quantification of carpaine in different effective sub fractions. For the present work, LCMS-MS method for quantitation of carpaine was developed, and validated.

3.7.1 Materials and methods

3.7.1.1 Chemicals

All analytical grade solvents and acids (methanol, n-butanol, glacial acetic acid, hydrochloric acid, sulfuric acid, chloroform, ethyl acetate, petroleum ether and ammonia) were purchased from Fisher Scientific Pvt. Ltd. (India). Bismuth nitrate and sodium sulphate were purchased from Sigma Aldrich (India). All solvents used for LCMS analysis were of LCMS grade and purchased from Sigma Aldrich (India).

3.7.1.2 LC-MS/MS

The chromatographic system consisted of Jasco quaternary pump, vacuum degasser (AB SCIEX Framingham, USA) coupled to QTrap® API-2000 mass spectrometer (Applied Biosystems, Foster City, CA, USA). LC separation was performed on XTerra® MS C18 column 50 mm×4.6 mm, 5.0 µm (Waters, Milford, MA, USA). The mobile phase consisted of 90% acetonitrile and 10% ammonium acetate (6 mM), pumped at a flow rate of 0.35 mL/min. Analysis time was 4 min per run.

API-2000 mass spectrometer equipped with ion spray source was employed for obtaining mass spectra. Data acquisition was carried out by analyst 1.4 software. Multiple reactions monitoring (MRM) mode was utilized to detect the compounds of interest. The mass spectrometer was operated in the positive ion mode for detection. The turbo ion spray setting and collision gas pressure were optimized (IS voltage: 5500 V, temperature: 350 ˚C, nitrogen was used as curtain and desolvation gas under following conditions nebulizer gas: 15 psi, curtain gas: 15 psi, GS1: 25 psi, GS2: 35 psi).

3.7.1.3 Method development for quantitation

Full scan product ion spectra of carpaine and piperidine as internal standard (IS) were recorded with MRM mode through the transition from precursor ion m/z 479.0 to product ion m/z 240.0. Linearity was determined by the analysis of standard solutions in the range of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, and 500.0 ng/mL using 100 mL of 1 µL/L piperidine (IS). Calibration curves were constructed by plotting peak area ratio (y) of carpaine to the internal standard, versus their concentration (x). Linearity was

accessed by weighted (1/x), linear regression of calibration curves generated in triplicate on three consecutive days using analyst-internal standard peak ratio. Parameters obtained from the calibration curve were used for calculating carpaine concentration in different parts of the *Carica papaya*.

3.7.1.4 Validation

Validation was performed according to the guidelines of International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use [84] and to recommendations of the *United States Pharmacopeia* (2009).
The lower limit of detection (LLOD) was determined at signal-to-noise ratio (S/N) of 3:1 and the lower limit of quantitation (LLOQ) was determined at a signal-to-noise (S/N) ratio of 10:1. Precision and accuracy were determined by computing percentage relative error (% RE) and percentage coefficient of variation (% CV) for intraday and inter day.

3.7.1.5 Statistical analysis

Values are expressed as mean ± SEM and data were analyzed using one-way ANOVA followed by the Dunnett's Multiple Comparison Test using GraphPad Prism (version 5; Graph- Pad Software Inc., San Diego, CA, USA). The significance level was set at $p < 0.05$.

3.7.2 Results and Discussion

Due to non chromophoric nature of carpaine it is difficult to analyse using PDA detector. Therefore development and validation of a quanitative analysis with LC-MS of carpaine was performed with a suitable internal standard (IS) *i.e.* piperidine.
For calibration curves, two mass transitions for carpaine were used (i.e. qualifier and quantifier) and one for IS resulted in well-shaped peaks at 1.73 min with good sensitivity (Figure 3.3).

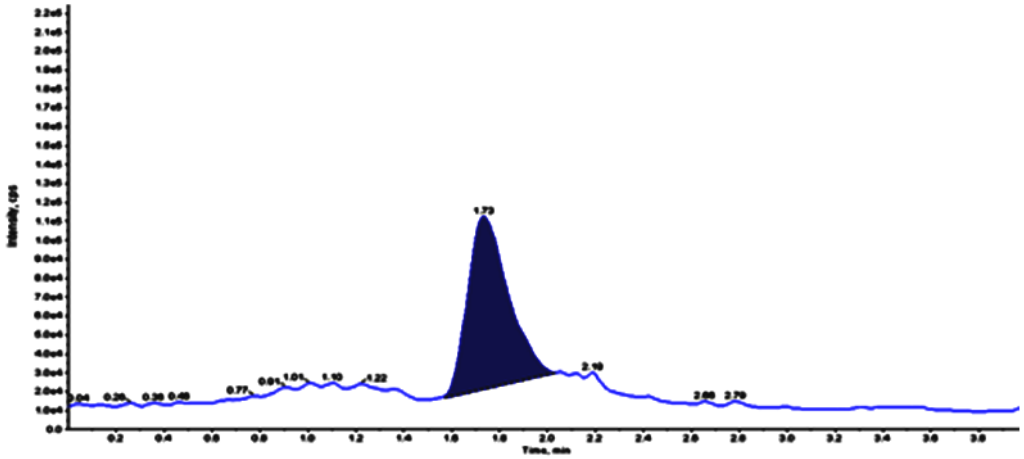


Figure 3.3: XIC chromatogram of carpaine spiked with piperidine as an internal standard (IS).

Relative response was calculated by dividing the analyte peak area to the internal standard peak area. The calibration curves were plotted for 479 m/z value corresponding to the parent ion and the product ion.

LOD and LLOQ were determined based on the MS response of a serial dilution of standard solution. LOD and LOQ were 0.1 ng/mL (S/N ≥ 3) and 0.5 ng/mL (S/N ≥ 10), respectively (Table 3.9). Good linearity was observed over the concentration range 0.5 to 500 ng/mL for carpaine (Figure 3.4).

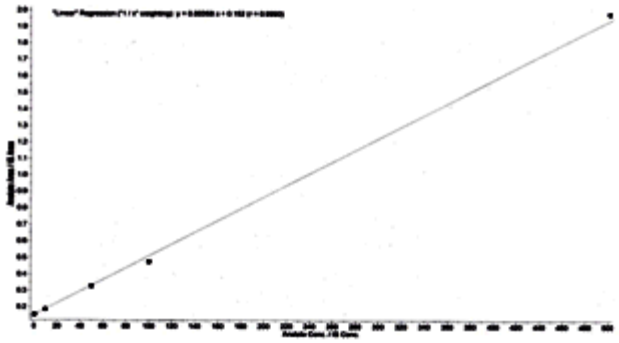


Figure 3.4: Calibration plot of carpaine

Table 3.9: Regression data, LOD, LOQ for carpaine

m/z	component	Regression	r^2	LOD(ng/mL)	LOQ(ng/mL)
479	Carpaine	y=0.00358x+0.152	0.999	0.1	0.5

The intraday (n=3) and inter day (n=3) analysis precision RSD % for intraday was 1.41 and precision RSD % for interday was 2.44. The accuracy (recovery) (n=3) was 97.3. All validation parameters reflect the good precision and accuracy of the method for quantification of carpaine.

The quantity of carpaine in water, petroleum ether and ethyl acetate extract was found to be 6.46 ± 0.09 mg/g, 28.56 ± 0.59 mg/g, 25.08 ± 0.53 mg/g respectively.

3.8 Conclusion

Based on the ethnopharmacological claim, *Carica papaya* was evaluated for its anti-thrombocytopenic activity. Although various reports suggested the anti-thrombocytopenic activity of *Carica papaya* but no systematic scientific studies were conducted on the chief chemical constituent responsible for this activity been identified [61], [62]. Thus, an attempt was made to identify the lead component responsible for the observed activity by a bioassay guided fractionation approach. The animal model of busulfan-induced thrombocytopenia was successfully developed and maintained. The alkaloid group consisting of carpaine, pseudocarpaine-I pseudocarpaine-II and choline [63] exhibited very good efficacy in maintaining platelet count. Thus these studies indicated that the alkaloidal group must comprise of bioactive component/s responsible for anti-thrombocytopenic activity.

In search of the main active component in alkaloidal group, it was further divided into two different groups, petroleum ether extract and ethyl acetate extract based on the polarity of the solvents. The screening of these two extracts showed very good anti-thrombocytopenic activity, with petroleum ether extract giving better activity over ethyl acetate extract. Finally the principal component of the alkaloid, carpaine, was isolated and tested for anti-thrombocytopenic activity which showed comparable activity with that of petroleum ether extract and ethyl acetate extract.

A pure sample of carpaine showed superior anti-thrombocytopenic activity than other groups of extracts. The isolated carpaine was identified and confirmed through different spectroscopic techniques as described in the next chapter 4.

It can be concluded that *Carica papaya* have good potential in maintaining the normal platelet count and may be used as an add-on approach for management of platelet count in thrombocytopenic patients induced under various conditions like dengue fever, chemotherapy treatment, etc. Furthermore, we have isolated and identified the chief chemical constituent responsible for this activity as carpaine. However, mechanistic studies need to be done to identify the underlying physiological pathway responsible for this observed activity. With respect to the toxicity studies, Ismail and coworker observed no toxicity for *Carica papaya* Linn. leaf extract in Sprague Dawely rats at a dose of 2 g/kg body weight [64]. Furthermore, to address the toxicological aspect of this study, hematological analysis, serum biochemical analysis and histological evaluation of liver, spleen and kidney of all the animals used in the experiment was carried out, after the termination of the study. The results obtained did not show any abnormalities in any of the animals and hence the data is not presented. However, detailed toxicity studies of carpaine need to be conducted to predict its safety in humans.

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