6.1 Summary

Dengue/dengue hemorrhagic fever has been one of the most imperative resurgent tropical diseases in the past 20 years, with escalating geographic allocation of viruses and mosquito vectors, amplified incidence of epidemics, the growth of hyperendemicity and the manifestation of DHF in new areas (Gibbons, 2010). DENV can cause a wide variety of clinical illnesses ranging from mildly symptomatic DF to more dangerous clinical conditions with capillary leakage syndrome such as DSS and DHF (Gubler, 2004). Each year, there are ~50 million DEN infections and ~500,000 individuals are hospitalized with DHF, mainly in Southeast Asia. DEN in India has dramatically expanded over the last few decades, with rapidly changing epidemiology. The first major DHF outbreak in the entire nation occurred in 1996 by DENV-2, and after a gap of almost a decade, the country faced yet another DF outbreak in the year 2003 by DENV-3 (Gubler, 1998; Wang et al., 2000; Koraka et al., 2001; Leong et al., 2007). At present, all the four serotypes are seen in circulation, but the predominant serotype keeps changing. Despite this trend, surveillance, reporting, and diagnosis of DEN remain largely passive in India. Traditional therapy for TCP consists of platelet transfusion, which can be linked with significant safety and economic issues. Consequently, efforts have been directed toward developing novel approaches for the treatment.

Leaves of Carica papaya L. has long been used as anti-inflammatory, antimicrobial and wound management (Goyal et al., 2009; Ansari et al., 2011). On administration of papaya in its powder form can significantly increase the PC of DEN infected patients (Sathasivam et al., 2009). The papaya extracts also shows the positive effects on other immune responsive cells like macrophages exhibiting antiviral properties (Mahbub-E-Sobhani et al., 2011; Du et al., 2011). The macrophages upon viral infection are responsible to produce antiviral antibodies. So this can be said that papaya may have many healthy effects on DEN infected patients due to its positive regulation of macrophages and platelets. The papaya leaves are rich source of α-tocopherol, ascorbic acid, flavonoids, phenolics, cynogenetic glucosides, and glucosinolates (Singhai et al., 2016)

The C. papaya L. leaves were collected, identified and authenticated. The tender leaves were cleaned, chopped and crushed to obtained juice. The juice was divided into four portions as CPLJ (500 mL), freeze dried powder (FDP; 1000 mL), bioactivity guided fractions (1500 mL) and metabolite enriched fractions (1000 mL). The % yields were calculated after drying and stored at -20°C until analysis. The freeze dried powder (FDP)
resulted in 4.84% w/w of mother extract i.e. CPLJ. The polarity based fractionation of papaya juice yielded 0.86% w/w of BBF of CPLJ. The metabolite enriched extraction and isolation of CRF, ARF and GRF was also done for total CPLJ. The FDP was used for the quality control analysis which includes total alkaloid, carbohydrate, glycoside, saponins, tannins, flavonoid, phenolics, protein and mineral content.

The chemoprofiling and marker constituent analysis using modern analytical methods especially chromatography was introduced by WHO for the quality assessment of the plant products and has been accepted worldwide as a tools for identification and quality control of herbal medicines (Farnsworth et al., 1985; WHO, 1992). LC-MS/MS, HPLC and HPTLC are the widely accepted analytical techniques for their high accuracy, precision and reproducibility of results whereas, HPTLC has many advantages because of low operating cost and less time consuming techniques (Larsen et al., 2004).

A simple, precise, accurate and simultaneous HPTLC, HPLC and LC-MS/MS method was developed for validation and quantification of myricetin, kaempferol, trans ferulic acid, para coumaric acid, caffeic acid and vanillic acid in CPLJ and in fractions, which is unique in itself and reported for the first time for analysis of flavonoids and phenolics, simultaneously. The method was validated as per the ICH guidelines (ICH Q2 (R1), for calibration, linearity, precision, accuracy, robustness, specificity, LOD, and LOQ. For quality control analysis of myricetin, kaempferol, trans-ferulic acid, para coumaric acid, caffeic acid and vanillic acid, these compounds are found in CPLJ which has been used in treatment of TCP (Dharmarathna et al., 2013; Zunjar et al., 2016; Anjum et al., 2017). These compounds have shown positive effect directly or indirectly in treatment of DF. Myricetin possess in-vitro anti-inflammatory activity and can reduce the acetic acid-induced capillary permeability (Wang et al., 2010). Kaempferol showed antimicrobial (Taechowisan et al., 2008) and strong anti-inflammatory activity (Hamalainen et al., 2007). Ferulic acid protects the body from neural disorders (Kanski et al., 2002; Srinivasan et al., 2007). Caffeic acid possesses a strong hepatoprotective activity (Itoh et al., 2009) was analysed using developed and validated LC-MS, HPLC and HPTLC methods (Parveen et al., 2014; Ahmad et al., 2015).

HPTLC analysis of FDP and fractions of CPL was performed on aluminum precoated plates of silica gel as stationary phase and toluene: ethyl acetate: formic acid (50:40:10; v/v/v) as mobile phase. The developed plates were air dried, and scanned at 320 nm revealed higher content of kaempferol in DBF and trans ferulic acid in BBF as compared to others fractions. A simultaneous HPLC method was developed and validated for the estimation of
myricetin, trans-ferulic acid, para coumaric acid, caffeic acid and vanillic acid in CPL. The mobile phase was optimized in such a way to give maximum resolutions between the components, which was critical to avoid merging/overlapping components and makes the method superior to other existing methods (Scartezzini et al., 2006; Pozharitskaya et al., 2007; Singh et al., 2008; Majeed et al., 2009). Further, the developed HPLC method was evaluated for different parameters as per the ICH guidelines and found accurate, reproducible, specific, and precise. HPLC analysis was performed on C18 column using the mobile phase of 0.5% formic acid (A) and acetonitrile (B) using PDA detector at wavelength 280 and 320 nm. The FDP and BBF revealed higher content of vanillic acid in BBF as compared to CPLJ. UPLC-qTOF/MS finger printing was performed on C18 column using the mobile phase of 0.5% formic acid (A) and acetonitrile (B), at a pH of 2.7, with gradient elution showed 25 compounds at different retention time out of which one was unknown, six phenolics, two alkaloids, and sixteen were hydroxycinnamic acid derivatives and flavonoids were tentatively identified. UPLC/MS chromatographic separation of analytes was carried out on Acquity UPLC HSS C18 (50 × 2.1 mm, 1.8 m) column using a gradient mobile phase consisting of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The analysis of mother extract and BBF revealed higher content of vanillic acid in BBF as compared to CPLJ. When compared to all the methods developed, LC-MS/MS was found best because of its sensitivity and sophistication gave good result in BBF having highest content of vanillic acid.

The safety profile studies of FDP was evaluated according to the Organization for Economic Co-operation and Development (OECD) guidelines 423 on Balb/c mice (25-30 g), where the limit test dose of 2000 mg/Kg was used for 14 days at three drug concentration for the selection of safe for in vivo screening and antithrombocytopenic activity. Animals were found safe at highest concentration. Pharmacological screening of different fractions was done on Balb/c mice for a period of seven days. All the fractions and the CPLJ were tested for platelet count (PC), total leukocyte count (TLC), bleeding time (BT), clotting time (CT) and activated partial thromboplastin time (aPTT) and were compared with the normal, toxic animals and animals received CPLJ. From the screening two best fractions were selected for in vivo antithrombocytopenic activity after inducing TCP using CP as negative control. BBF and CRF were found more potent than CPLJ. The BBF and CRF significantly enhanced the production of platelets (PC; p < 0.05), the CPLJ also showed little increment in platelet levels, while FDP and other fractions showed increment in PC but not as significant as BBF.
and CRF. The TLC and aPTT showed significant difference (p<0.05) between the different treated groups. The BBF showed highly significant (p<0.05) increase in PC, TLC (p<0.05) and aPTT (p<0.05), when compared with CPLJ and FDP. The fractions were also screened for CT and BT that were found significant (p<0.01) in BBF and CRF, when compared with normal control animals on day 0, day 3 and day 10, respectively. The selected fractions were tested for antithrombocytopenic activity for a period of 21 days after inducing TCP using CP and the fractions were compared with negative control, positive control and the parse group (only received CPLJ). The BBF showed highly significant (p<0.05) increase in PC, TLC (p<0.05), aPTT (p<0.05), PT (p<0.01), IgG and IgM (p<0.05) when compared with negative control treated with 200 mg/kg b.wt cyclophosphamide. The mean PC and TLC mice treated with BBF were significantly higher (p<0.05) on day 21 as compared to day 4 after inducing TCP using CP, respectively. The groups were compared with negative control receiving CP (200 mg/Kg body wt.). Study found that CPL extracts inhibits heat-induced and hypotonicity-induced hemolysis of erythrocytes obtained from both healthy individuals and individuals with DEN infection; the effect was observed at the lower concentrations of the extracts. Thus, it possesses membrane-stabilizing properties and protects blood cells against stress-induced destruction. It is postulated that the effect could be due to the presence of flavonoids and other phenolic compounds in the papaya leaves.

From the in vivo thrombocytopenic activity BBF gave the best result and hence selected further for quantification and validation of markers in rat plasma by HPLC and LC-MS/MS. The sensitivity of the methods were determined by limit of detection (LOD) and lower limit of quantification (LLOQ) using the calibration curve method, according to the ICH recommendations. The LLOQ can be accurately quantified within a 20% bias of the nominal concentration and with a precision not exceeding 20% in plasma.

The HPLC separation was performed using a C18 column (100 × 2.0 mm, i.d. 3.0 μm) with the gradient mobile phase at a flow rate of 1.0 mL/min. UV detection was carried out at a wavelength of 280 and 320 nm and the whole analysis took 25 min. Protein precipitation in plasma samples was performed using methanol for both HPLC and LC-MS. The method was found linear in the wide range with the correlation coefficient for each analyte more than 0.992. The limit of detection (LOD) and lower limit of quantitation (LLOQ) were also detected. Intra- and inter-day precision and accuracy (RSD) were less than 3%. The LC-MS/MS method was also developed and validated to determine the six constituents in vivo and to conduct pharmacokinetic studies on it. Myricetin, kaempferol, trans ferulic acid, para...
coumaric acid, vanillic acid and caffeic acid were analysed by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). The analytes were separated on a BEH C18 column and quantified by multiple reactions monitoring (MRM) mode. The linear regression showed high linearity over a 06-100 ng/mL dynamic range for the six analytes. The relative standard deviations of intra- and inter-day measurements were less than 8.50%, and the method was accurate. The extraction recoveries were found in range 84.37 to 90.48%. All samples were stable under short- and long-term storage conditions.

The validated method was successfully applied to a comparative pharmacokinetic study of myricetin, kaempferol, trans ferulic acid, para coumaric acid, vanillic acid and caffeic acid in BBF as their free and total forms in rat plasma. The non-compartmental pharmacokinetic analysis of the concentration-time data was performed using Kinetica 5.1. There were perceptible differences in pharmacokinetics parameters (C\text{max}, AUC\text{0→t}) of the analytes. Developed and validated simultaneous method produced more than 85% of recovery of the targeted metabolites in plasma through HPLC and LC-MS. The mean plasma concentration versus time profiles was found highest in myricetin and the half-life and AUC\text{0→t} was found highest for vanillic acid, respectively by HPLC and LC-MS. Although the administered doses of caffeic acid, para-coumaric acid, trans-ferulic acid and kaempferol were rather lower than those of the myricetin and vanillic acid, the AUC\text{0→t} of vanillic acid was higher than those of trans-ferulic acid, caffeic acid, etc., which demonstrates that the bioavailability of the hydroxycinnamic acid derivative was much lower than that of the dihydroxybenzoic acid derivative. The T\text{1/2} of caffeic acid was higher in BBF, which indicates that the dihydroxybenzoic acid derivative is metabolized significantly higher than the dihydroxycinnamic acid derivatives \textit{in vivo}. In addition, AUC\text{0→t} and C\text{max} values obtained for trans ferulic acid and vanillic acid were significantly higher than those obtained from the other flavonoids. This finding demonstrates that the phenolics were more easily absorbed into the blood and more quickly metabolized compared with the flavonoids.

The targeted and untargeted metabolic profiling of BBF and fate of metabolites present in BBF after its oral administration has been identified by comparing chromatograms i.e. peak pattern of BBF as well as blood plasma. Mass spectrum of every metabolites separated through LC-MS were tentatively identified by comparing m/z values and literature survey along with verifying with publicly available database. A total of 350 metabolites were
screened by LC-MS chromatograms of BBF and plasma showing the number of metabolites separated by LC-MS.

Further the quantified compounds (as ligands namely myricetin, kaempferol, trans-ferulic acid, para coumaric acid, caffeic acid and vanillic acid) in BBF were docked with different target enzyme namely thrombopoietin, α2 adrenergic receptor, Integrin αIbβ3, Integrin α2β1, cMPL, GPIb/IX/V, P selectin, P₂Y₁₂ and P₂Y₁. Best docked pose was shown by flavonoids giving high binding energy. Hence, the present study shows that bioactive guided butanol fraction of CPLJ might increase thrombocytes in DF related TCP.

6.2 Conclusion

For the standardization of CPLJ and its fractions, new analytical methods were developed and validated for estimation of marker constituents which can be used for the quality control of the crude drugs and formulations containing CPLJ and its fractions as ingredient. The developed methods were HPTLC, HPLC and LC-MS methods and used for the simultaneous estimation of myricetin, kaempferol, trans ferulic acid, para coumaric acid, caffeic acid and vanillic acid for the first time (Anjum et al., 2017).

The safety profile studies were performed on FDP on Balb/c mice for 7 days and were assessed physically and biochemically. It was found safe at higher dose upto 2000 mg/Kg body wt. The CPLJ, FDP and their different 7 fractions were screened for in-vivo antithrombocytopenic activity without inducing thrombocytopenia in mice. From the screening best fraction was selected for in vivo antithrombocytopenic activity for a period of 21 days after inducing thrombocytopenia using cyclophosphamide as toxicant. Cyclophosphamide cause lowering of platelet count in blood, its effect the immunity which was evident as it increased serum ALT and AST; platelet count was reduced to extremely low level so as TLC also. Bleeding and clotting time was also drastically affected by this toxicant.

The BBF offered positive effect on serum AST and ALT level, platelet count and TLC count was increased whereas BBF did reduced aPTT, PT, BT and CT in affected mice. IgG and IgM level were also monitored in mice. Overall, BBF offered good overall protection against cyclophosphamide induced thrombocytopenia and showed significant reversal on PT and TLC when compared to toxicant group.

Results were further supported by pharmacokinetics and in silico docking studies by studying its binding with 9 different functional platelet receptors. Hence, it can be concluded that out of the nine fractions screened from preliminary antithrombocytopenic activity,
bioactive butanol fraction showed significantly better thrombocyte enhancement in dengue fever related thrombocytopenia.

The findings of the present study strongly suggest that there could be some active compounds in BBF that enhances hemopoiesis and thrombopoiesis in animals. The quantitative analysis results provide scientific and comprehensive information for the quality control of BBF thereby selecting for administration in dengue fever related thrombocytopenia. Chemical analysis of *C. papaya* L. leaves showed the presence of syringic acid, guaifenesin, myricetin, kaempferol, trans-ferulic acid, caffeic acid, vanillic acid, para coumaric acid and unidentified constituents. The results showed good impact on mice by four folds increase in PC, which was evidently confirmed by studying aPTT and PT. The pK analysis proved good bioavailability of vanillic acid, myricetin, trans-ferulic acid and kaempferol. Based on our observations of *in silico* analysis, the flavonoids myricetin and kaempferol in BBF proved to have good binding with thrombopoietin, α2 adrenergic receptor, Integrin αIIβ3, Integrin α2β1, cMPL, GPIb/IX/V, P selectin, P2Y12 and P2Y1 receptors and might exert thrombocyte enhancing property. Further investigations are necessary to identify the active compounds in BBF which are responsible for the activation of hemopoiesis and thrombopoiesis.

However, analyzed and studied CPLJ and BBF have shown good antithrombocytopenic potential in tested animal model and same has already been proved by various authors in clinical trials. The metabolomic potential of the CPLJ and BBF has resulted in identification of major metabolites present in them. Further quantitative estimation followed by PK studies of more than four bioactive components as proved by *in silico* analysis is evident enough to consider them as a phytopharmaceutical.

In conclusion, the study is very encouraging to develop the CPLJ and or BBF as effective antithrombocytopenic/antidengue phytopharmaceutical. However, neurotoxicity, genotoxicity, stability and shelf life studies are needed to follow up this project and to launch this drug in the market as a phytopharmaceutical product. The studies on anti-DENV 1-4 serotypes may also be planned to find its antivirus potential that will provide a proof for this drug as a complete regimen against DENV.