

Effect of Purified Alkaloid (Carpaine) From Water Extract of Carica Papaya L. Leaves on Platelet Count of Carboplatin Induced Thrombocytopenic Sprague Dawley Rats

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ARTICLE INFO

Article History:

Accepted : 01 July 2025

Published: 03 August 2025

Publication Issue :

Volume 12, Issue 4

July-August-2025

Page Number :

886-894

ABSTRACT

Current research work is focused on purification of carpaine from water extract of Carica papaya L. leaves, its analyses and its effect on hematology and histopathology of carboplatin induced thrombocytopenic Sprague dawley rats. Functional group detection tests, FTIR and LCMS analyses confirmed the presence of carpaine. Carboplatin induced thrombocytopenic Sprague dawley rats were dozed with purified carpaine (2mg/kg, and 4mg/kg body weight). Positive effect of purified carpaine was observed on platelet count while its effect on other hematological parameters (Hb, RBC, WBC, PCV, MCV, MCHC, MCH, heterophiles, eosinophils, lymphocytes and monocytes) was not note-worthy. Histopathology of kidney, liver, lungs, heart, intestine and spleen revealed no lesions, toxic effects and any abnormality. Thus from the results it can be concluded that carpaine could be the potent biocomponent present in Carica papaya L. leaves responsible for increasing platelet count.

Keywords: Carica papaya L., thrombocytopenia, platelet count, carpaine

I. INTRODUCTION

Thrombocytopenia is a condition associated with the lower production of platelets than the normal numbers in the bone marrow and is often multifactorial. Diseases such as dengue, idiopathic thrombocytopenic purpura (ITP), malignancy, aplastic anemia, drug induced thrombocytopenic purpura (for example Heparin induced thrombocytopenia), hemolytic uremic syndrome etc. result in a low thrombocyte count in blood (Aster R. H. and Bougie D. W., 2007).

Thrombocytopenia is defined as a platelet count below the 2.5th lower percentile of the normal platelet count distribution. The physiological range for thrombocytes in the normal healthy human is $150-400 \times 10^9/l$ of blood. Thrombocytopenia causes few signs or symptoms with platelet count in the range of $100-150 \times 10^9/l$ that represents mild thrombocytopenia. Platelet count of $50-100 \times 10^9/l$ and levels less than $50 \times 10^9/l$ is categorized as moderate and severe thrombocytopenia respectively (Wong W. and Glader B., 2004).

Thrombocytopenia is caused by two basic reasons; one is impaired platelet production and second is excessive destruction. Impaired platelet production occurs in bone marrow (BM) failure, megaloblastic anemia, leukemia, myeloma, myelo-fibrosis, solid tumor infiltration, aplastic anemia and paroxysmal nocturnal haemo-globinuria (PNH). Excessive destruction occurs in autoimmune ITP, Systemic lupus erythematosus (SLE), viral infections like Epstein-Barr virus (EBV), dengue and human immunodeficiency virus (HIV), disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP) and in hyper-splenism (Tahir N. et al, 2014).

The water decoction/extracts of leaves of *Euphorbia hirta*, guava leaves, *Carica papaya* L., *Ipomea batatas* green and violet variety, *Alternanthera sessilis*, *Euphorbia hirta*, Beetroot and Red guava are used as a folk medicine to increase platelet count in case of dengue fever, *Psidium guava* ripe fruit or juice are noted to elevate platelet count (Parida M. M. et al, 2002, Arollado E. C. et al, 2013, Abouthealth.com/dengue-fever-medicine, 2011, Jhansi Rani M., et al, 2015).

The juice of *Carica papaya* L. leaf has been observed to enhance platelet count in randomized clinical trials by various researchers with different number of subjects (Deepak B. S. R. et al, 2013, Subenthiran S. et al, 2013, Naresh Kumar C. V. M. et al, 2015, Bajaj P. and Sharma R., 2016). *Carica papaya* L. leaves extract exhibited potential to increase platelet count to fight thrombocytopenia (Hettige S. et al, 2008, Nisar A. et al, 2011, Naresh Kumar C. V. M. et al, 2015, Kasture P. N. et al, 2016). The *Carica papaya* L. leaf aqueous extract at various concentrations were found to increase the platelet count significantly in thrombocytopenic rat model (Sathasivam K. et al, 2009, Patil S. et al, 2013, Tahir N. et al, 2014). The study of Gammulle A. et al, (2012) reiterated that the mature leaf *Carica papaya* L. concentrate (MLCC) has the potential to be developed as a plant based therapeutic agent for thrombocytopenia.

World-wide *Carica papaya* L. leaves extracts are gaining popularity as folklore medicine for the treatment of different types of thrombocytopenia. In spite of several scientific experiments carried out on animals and humans, negligible knowledge about the bio-active compounds responsible for increasing platelet count is available.

The probable active components of *Carica papaya* L. leaf extracts for increasing platelet count include enzymes such as papain, chymopapain, caspase; secondary metabolites like carpaine, pseudo-carpaine and dehydro-carpaine I and II, flavonoids, benzyl-glucos-inolate and tannins; Vitamins - B12, C, and D; minerals - calcium, magnesium, potassium, and sodium (Gammulle A. et al, 2012, Tahir N. et al, 2014, Julianti T. et al, 2014, Zunjar V. et al, 2014).

The exploration of exact composition of bio-components or individual bio-component(s) responsible to raise the platelet count and counter act thrombocytopenia is not yet identified.

II. METHODS AND MATERIAL

2.1 Purification of alkaloids:

Water extract of shed dried leaves of *Carica papaya* L. was obtained by macerating the powder in distilled water and then kept on shaker (ratio 25:100) for 8 hours. The extract concentrated using rotary vacuum evaporator (evaporator) was used for isolation of carpaine. Fourteen days soaked (94.5:5:0.5, EtOH/DW/GIAA) extract (25 g) filtered through Whatmann filter paper no.1 and centrifuged at 5000 rpm for 15 min. Supernatant concentrated to a thick dark brown slurry using rotary vacuum evaporator at 60°C. pH of the slurry treated with DW: EtOH (49:1) and diethyl ether (50 ml) was adjusted to pH 11 with potassium carbonate solution and then re-extracted with diethyl ether (50 ml). The ether extract was washed with distilled water (50 ml) and 5% hydrochloric acid solution (50 ml) was added. The pH of acidic aqueous layer obtained again adjusted to 11. Final ether extract was dried with sodium sulphate (anhydrous), and

diethyl ether was removed at 30°C. The brown slurry obtained yielded a precipitate of dull yellow needle-like carpaine crystalized upon chilling at -20°C in a Bio-freeze laboratory refrigerator (Faizal M. B. M. and Abdul N. 2014).

Crystals of carpaine were separated by treating dull yellow and brown crystals with hexane at 40°C. Further to obtain white crystals of pure carpaine it was washed with distilled water and treated with acetone at 40°C (Zunjar V. et al, 2014).

2.2 Qualitative tests for detection of functional groups:

To predict the identity of purified compound, functional groups detection tests were performed as per the methods given by Svehla G., (1979) with slight modifications.

2.3 FTIR Analysis:

Purified carpaine samples and potassium bromide were taken in 1:3 ratio and made into fine powder. The samples were analyzed as per instructions on FTIR (AR Affinity-1, Shimadzu) for the presence of functional groups.

2.4 LCMS Analysis:

HPLC/LCMS analysis of the crystals of purified sample was carried out at CDRI, Lucknow (India) as per SAIF-CDRI Lucknow Standards on Waters Xevo TQD triple quadrupole mass spectrometer (USA). It was equipped with waters, Alliance 2695 HPLC system and electrospray ionization source. The HPLC column used was SUNFIRE C-18 column (250 mm × 4.6 mm id, 5µm) and dual mode (+) LC-ESI-MS experiments performed after injecting 20µl samples through auto-sampler. Mobile phase used was 0.1% formic acid aqueous solution and acetonitrile. A linear gradient elution was performed at the flow rate of 1.5 ml min⁻¹. Nitrogen was used as the nebulizing and drying gas at flow rates of 50 and 950 L h⁻¹ respectively.

2.5 Animal Trials:

Procured both male and female Sprague dawley rats were housed in microloan boxes in a controlled hygienic environment at temperature 25°C±2°C and 12hr dark/light cycle. The study was conducted after

obtaining institutional animal ethical committee's clearance.

A set of 24 Sprague dawley rats was divided into four groups of 06 (03 Males and 03 females) animals each. On 1st day, platelet count of the rats was lowered down by giving IP injection of Carboplatin (50mg/kg body weight). On 3rd day, two groups were given purified alkaloids obtained from water extract (2mg/kg and 4mg/kg body weight) dissolved in carboxy-methyl-cellulose solution (2%), while third group was given single dose of standard drug prednisilon (Intervet India Pvt Ltd) (5mg/kg) to increase platelet count. Fourth group was allowed to recover naturally. Retero-orbitally blood sample was withdrawn on day 1, 3, 5, 7 and 10 for hematological studies.

2.6 Hematological studies:

Hematological parameters (Hemoglobin, RBC, WBC, Platelets, Packed Cell Volume, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin Concentration, Mean Corpuscular Hemoglobin, Heterophile, Eosinophils, Lymphocytes, Monocytes) were studied as per the methods given by Godkar P.B. and Godkar D.P. (2003).

2.7 Histopathological studies:

At the end of experiment animals were sacrificed for histopathological examinations of Liver, lungs, kidney, spleen, heart and intestine to check effect of extract on organs and its toxicity were done as per the methods given by Godkar P.B. and Godkar D.P. (2003).

III.RESULTS AND DISCUSSION

3.1 Purification of Carpaine:

Partial purification of alkaloids from water extract of Carica papaya L. leaves was done by the method given by Faizal M. B. M. and Abdul N. (2014). Partially purified carpaine crystals were dull yellow and needle like. The amount of partially purified alkaloids obtained in water extracts was 13.41%.

Table No. 1: Yield of Alkaloid in water extract

Sr. No.	Sample/Extract	Weight taken (gm)	Yield (gm)	% yield
1	Water	07	0.939	13.41

**Figure No. 1: Crystals of partially purified alkaloids from Water extract**

Partially purified carpaine crystals were further purified by the method given by Zunjar V. et al, (2014). Purified carpaine crystals were white and the yield obtained was 7.14%.

**Figure No. 2: Purified carpaine crystals****Table No. 2: Yield of pure alkaloid**

Sr. No.	Sample/Extract	Weight (gm)	Yield obtained (gm)	% yield
1	Water	7	0.5	7.14

Zunjar V. et al, (2014) had obtained varying concentration of carpaine 16.79 ± 70.09 mg/g, 117.10 ± 70.59 mg/g, 99.47 ± 70.53 mg/g in water,

petroleum ether and ethyl acetate extract of *Carica papaya* L. leaves respectively. Thus, alkaloid (Carpaine) content in *Carica papaya* L. leaves found in range of 1.6% to 11.70%.

3.2 Qualitative tests for detection of functional groups:

Crystals obtained were subjected to functional group detection tests as mentioned in the method given by Svehla G., (1979) with slight modifications. Positive results obtained for unsaturation, secondary amines, ketones (carbonyls) and esters are depicted in table no. 3.

Table No. 3: Functional group detection tests of purified alkaloids

Sr.	Test	Observation	Inference
1	Unsaturation	De-colorization	Compound was unsaturated
2	Secondary amines	No precipitation	Secondary amines were present
3	Ketones (carbonyl)	Red color was not formed	Ketone (carbonyl) group was absent
4	Esters	De-colorization was observed	Ester group present

3.3 FTIR Analysis:

FT-IR spectra obtained were analyzed and interpreted for functional groups present in purified carpaine by the method described by Kalsi P. S., (2004). IR stretching and frequencies obtained in purified carpaine were in good harmony with the standard frequencies.

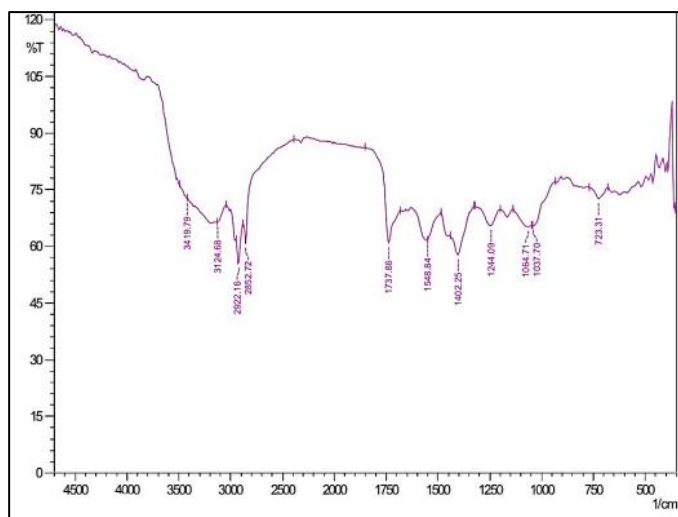


Figure No. 3: FT-IR bands of purified carpaine

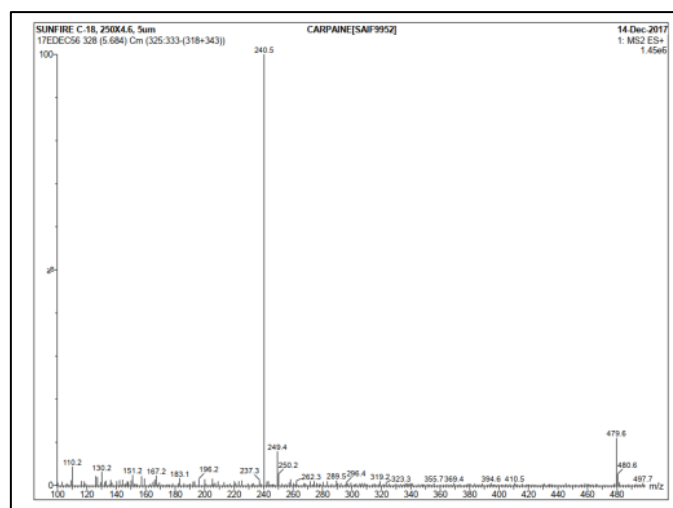


Figure No. 5: LCMS PEAK

Table No 4: FTIR analysis of purified carpaine

Observed cm ⁻¹ Carpaine	Frequency in purified Carpaine	Bond stretching
3419, 3124		N-H Stretching in sec. amines
2922, 2852		C-H Stretching in methyl group
1737		C=O Stretching in ester
1548, 1402		C-C Stretching in cyclic ring
1244		C-N Stretching in Amines
1064		C-O Stretching in esters

3.4 LCMS Analysis:

Purified alkaloid crystals were analyzed by LCMS at CDRI Lucknow. Figure indicates the MRM and m/z pattern of the same.

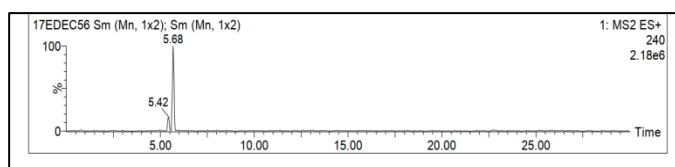


Figure No. 4: MRM Peaks

Full scan product ion spectra of carpaine was recorded with MRM mode through transition from precursor ion m/z 479.0 to product ion m/z 240.0 by Zunjar V. *et al*, (2014) and Julianti T. *et al*, (2014). As the standard was not available in market, the previous published data was used as reference.

In the current research LCMS analysis of purified alkaloid crystals also showed precursor ion m/z 479.6 and product ion m/z 240.5, which confirmed the presence of carpaine.

The Ultra Performance Liquid Chromatography Triple Time-Of-Flight Electrospray Interface Mass Spectrometry (UPLC-TripleTOF-ESI-MS) fingerprinting of *C. papaya* L. leaf was done by Adlin A. *et al*, (2012). The fingerprinting exhibited 17 peaks, which were resolved within 6.5 min. The retention times, pseudomolecular ion and fragmentation ions of all the compounds were detected in the extract. Peak no. 17 exhibited [M+H]⁺ at 479 and the fragmentation ions at m/z 240 [half molecular + H]⁺. Similar ESI data had been reported by Bennet R. N. *et al*, (2004) and Jiao Z. *et al*, (2010) and mentioned the alkaloid as carpaine. The isolated carpaine was identified through different spectroscopic techniques (FT-IR and LCMS).

3.5 Hematology:

Hematological data were evaluated statistically with MINITAB version 16 software. Data was analyzed using Kruskal-Wallis test. Positive effect of purified carpaine was observed on platelet count while its

effect on other hematological parameters (Hb, RBC, WBC, PCV, MCV, MCHC, MCH, heterophiles, eosinophils, lymphocytes and monocytes) was insignificant so not mentioned here. The comparison showed that mean platelet count in water extract was significantly higher than control group ($P < 0.05$).

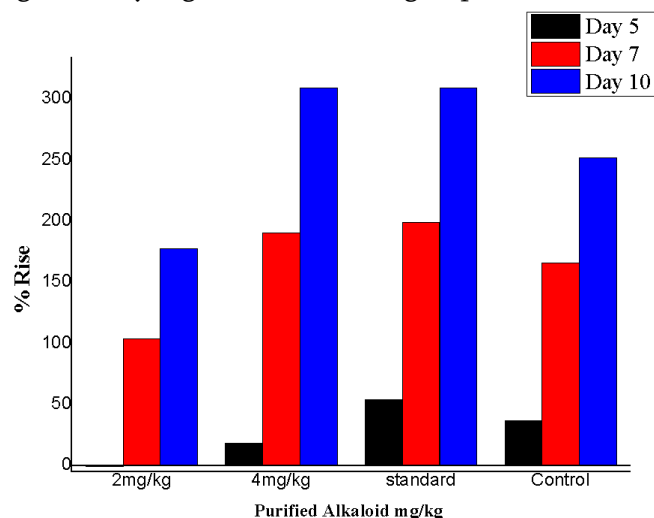


Figure No. 6: Effect of purified carpaine on platelet count

Table No. 5: Effect of purified carpaine on platelet count

Day 1				
Particular	Group 1 Carpaine (2mg/Kg)	Group 2 Carpaine (4mg/Kg)	Group 3 Standard (5mg/Kg)	Group 4 Control*
PLT×105/cmm before induction of thrombocytopenia	8.61±0.77	8.42±1.63	8.81±1.03	8.63±0.99
After blood withdrawal thrombocytopenia was induced using single dose of carboplatin				
Day 3				
PLT×105/cmm after induction of thrombocytopenia	1.78±0.84	1.49±0.81	1.85±0.84	1.12±0.16
% decrease in PLT count after induction of thrombocytopenia	79.33	82.30	79.00	87.02
After blood withdrawal single dose of carpaine/prednisilon was given				
Day 5				
PLT×105/cmm after single dose of carpaine/ prednisilon	1.77±0.55	1.76±0.66	3.71±1.17	1.54±0.44
% increase in PLT count as compared to day 3	-0.56	18.12	100.54	37.50
Day 7				
PLT×105/cmm after single dose of carpaine/ prednisilon	3.63±1.34	4.33±1.81	7.20±0.79	3.00±0.30
% increase in PLT count as compared to day 3	103.93	190.60	289.19	167.86
Day 10				
PLT×105/cmm after single dose of carpaine/ prednisilon	4.94±0.87	6.10±1.37	9.87±1.27	3.97±0.42
% increase in PLT count as compared to day 3	177.53	309.40	433.51	254.46

* Control group rats were not given carpaine/prednisilon and were allowed to recover naturally

On 5th day the platelet count rise was observed by - 0.56%, 18.12%, 53.94% and 36.48% in case of 2mg/kg body weight, 4mg/kg body weight, standard and control groups respectively. This shows that, even more drop in platelet count (79.44%) was found on 5th day in case of 2mg/kg body weight. The dose might not be that effective as compared to 4mg/kg body weight on the same day.

The rise in platelet count on 7th and 10th day was more by 83.39% and 74.28% respectively in case of 4mg/kg body than 2mg/kg weight dose. The results were found to be graphically and statistically significant in the dose of 4mg/kg body weight of purified alkaloid.

Anti-thrombocytopenic activity was evaluated on busulfan induced thrombocytopenic Wistar rats by Zunjar V. et al, (2014). The anti-thrombocytopenic activity of different fractions of extracts was evaluated by monitoring blood platelet count. Animals were treated with carpaine at a dose of 2 mg/kg for 20 days. Carpaine maintained platelet count till $555.55 \pm 27.22 \times 10^9/l$. Carpaine, isolated from the alkaloid fraction exhibited potent activity in sustaining platelet counts with no acute toxicity. Study scientifically validated the usage of *Carica papaya* L. leaves and it also proved that alkaloids particularly carpaine present in the leaves to be responsible for the anti-thrombocytopenic activity. The results of experiments suggested that anti-thrombocytopenic property of *Carica papaya* L. leaves may be attributed to carpaine dose.

3.6 Histopathology:

At the end of experiment, animals were sacrificed for histopathological examinations. Histopathological examination of the liver, lungs, kidney, intestine, heart and spleen of standard, control and treated groups did not reveal any morphological differences or lesions.

From histopathological studies it is clear that all doses of purified alkaloid had no toxic effects on major organs like liver, lungs, kidney, spleen, heart and intestine. Hence the purified alkaloid (Carpaine) is safe by oral route for increasing platelet count.

Similarly, no toxic effects of carpaine on liver, spleen and kidney were observed by Zunjar V. *et al*, (2016).

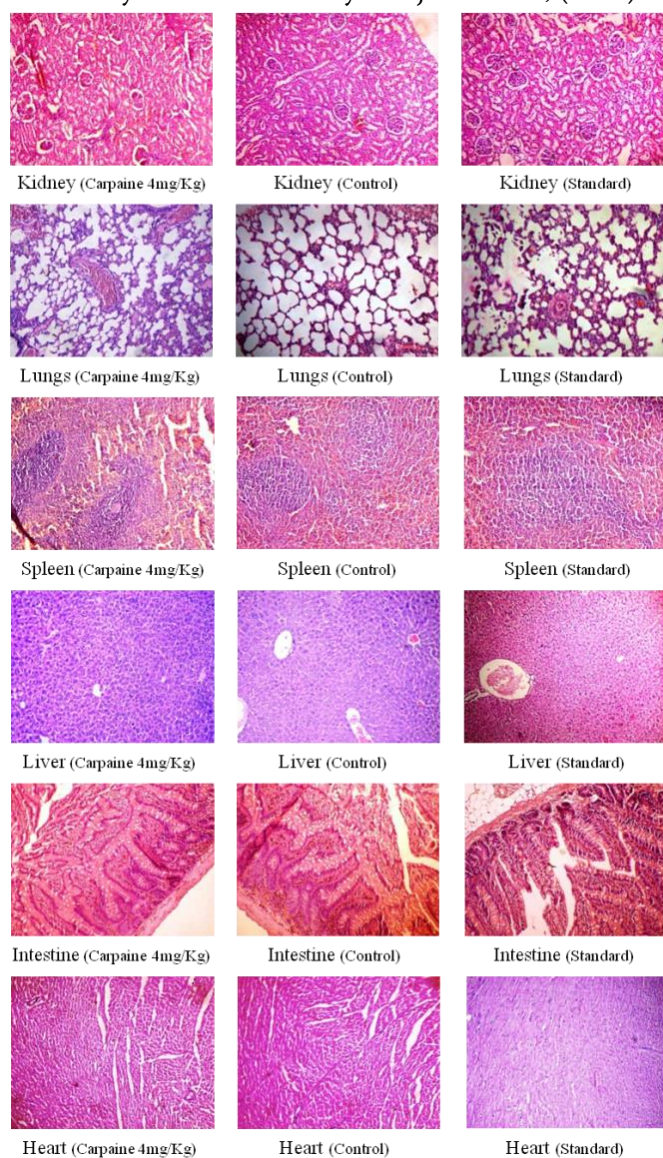


Figure No. 7: Histopathological images of the various organs

IV. CONCLUSION

Thus from the above findings, it can be concluded that *Carica papaya* L. leaves in the form of juice or extract can be used in the treatment of thrombocytopenia. The potent bio-component present in papaya leaves i.e. alkaloid- Carpaine could be a good therapeutic agent in treatment of thrombocytopenia.

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