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Phytochemical screening and evaluation of platelet stimulating activity of carica papaya leaf ethanolic extract

Abstract

Carica papaya is an herbaceous plant belonging to the family *Caricaceae*. The leaves of this plant were collected from Pune area, Dried, Powered and then extracted with ethanol. Phytoconstituents were isolated by the treating with NaOH and HCL, and further get separated by column chromatography. Structural elucidation of mainly two column fractions was carried out using GCMS and HRMS analysis. Fraction one and fraction two showed presence of Pthalic acid ester derivatives and carboxylic acid derivatives and some unknown compounds respectively. All the samples were tested for platelet stimulating activity on Hydroxyurea induced Thrombocytopenia rat model. The changes in hematological parameters were measured. After giving a treatment of crude ethanolic extract, Fraction one and Fraction two, the blood was withdrawn from the retro-orbital plexus and tested for the hematological parameters, in which more significant increased in the platelet number and other hematological parameters were observed for Fraction one i.e Phthalic acid ester derivative in a time-dependent manner. The current study supports platelet augmentation activity of Carica papaya leaf ethanolic extract and its active constituent phthalic acid ester derivative, which can have a beneficial effect in thrombocytopenia.

Keywords: carica papaya, thrombocytopenia, platelet stimulation, hydroxyurea

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Introduction

Carica papaya (papaya, papita, pawpaw) is an herbaceous plant belonging to the family Caricaceae. The plant is recognized by its weak and usually unbranched soft stem yielding copious white latex and crowned by a terminal cluster of large and long-stalked leaves. It is rapidly growing and can grow up to 20m tall.[1]Traditionally, leaves have been used for the treatment of a wide range of ailments, such as malaria, dengue, and jaundice, immune-modulatory,and antiviral activity. Young leaves are rich in flavonoids (kaempferol and myricetin), alkaloids (carpaine, pseudocarpaine, dehydrocarpaine I and II), phenolic compounds (ferulic acid, caffeic acid, chlorogenic acid), and the cytogenetic compounds (benzyl-glucosinolate) found in this leaf. Both the leaf and the fruit of the Carica papaya Linn.[2] possess carotenoids, namely β- carotene, lycopene, anthraquinones glycoside, and hence possess medicinal properties like anti-inflammatory hypoglycemic, anti-fertility, abortifacient, hepatoprotective, and wound healing.Recently its antihypertensive and antitumor activities have also been established. Leaves being an important part of several traditional formulations are undertaken for standardization for various parameters like moisture content, extractive values, ash values, swelling index, etc. It also has anti-cancer[3] activities, anti-hypertensive,[4] immunostimulant,[5] anti-sickling,[6] and anti-fertility activities. Carica papaya is also used in the treatment of ulcers,[7] diabetics,[8] etc. Platelets are the type of blood cells produced from megakaryocytes by the action of a major hormone Thrombopoietin.[9] Colony stimulating factor (CSF) and cytokine-like IL-3, Il-6, and IL-11 also play their role in the thrombopoiesis.[10] Platelets are mainly involved in the blood clotting mechanism to control the blood loss during injury along with other blood coagulation factors. Thrombocytopenia (Low platelet count) is a serious life-threatening condition and is either due to decreased production of platelets from bone marrow or increased destruction of platelets due to an immune reaction.[11] Thrombocytopenia is mainly linked to the prolonged bleeding and clotting time due to an insufficient number of platelets in blood.[12] The decrease in normal platelet count is mainly caused by genetic disorders or infections like dengue hemorrhagic fever, chickenpox, parvovirus, rubella, and several bacterial infections like tuberculosis.[13] Most chemotherapeutic agents can cause thrombocytopenia as an adverse effect during the period of treatment. Based on this effect, hydroxyurea (Hyu) was used to induce thrombocytopenia in experimental animals for the evaluation of platelet stimulation activity of the prepared extract. The main objective of the present study is to evaluate the platelet stimulation activity of Carica papaya leaf ethanolic extract (CPE) and its isolated constituent phthalic acid ester derivative and octadecanoic acid ester derivative by using HYu induced thrombocytopenia model in Wistar albino rats. Effect of prepared extract on RBC, WBC, Hb and Hematocritwas also studied simultaneously.

Material and methods

Plant material and drug

Carica papaya fresh aerial parts i.e leaves were obtained from Chakan, Pune, India, in the month of September 2015. Leaves were dried at room temperature. The plant was identified and authenticated as Carica papaya (L) Family*: Caricaceae*, from a botanical survey of India, Pune by Dr. P. Lakshminarasihan, Research officer, Voucher specimen no. (BSI/WRC/Iden./2015/409). Hydroxyurea capsule (Hydrea) was purchased from the local medical shop, Chakan.

Animals

Adult female Wistar rats (180-250 g) were procured from the authenticated supplier, In Vivo Bioscience, Pune, Maharashtra, India. All animals were housed under standard laboratory conditions, maintained on a 12 hrs light: 12 hrs dark cycle. Food and water were provided. Animals were acclimatized for 7days to laboratory

conditions before the test. After 7days, the animals were divided into 6 groups.

Powder characteristic study of plant material

The crude drug powder was observed for the presence of macroscopical parameters i.e. color, odor, taste, determination of foreign matter, ash value,[14] acid insoluble ash, water soluble ash, loss on drying, etc.

Preparation of crude ethanolic extract

The powdered leaves were used for extraction by using hot continuous extraction process i.e. Soxhlet extraction.[15] With Ethanol (95%) in a Soxhlet extractor for 8 hrs at temperature 55^{0}c. The obtained extract was weighed and its percentage in terms of air-dried weight of the plant material was calculated. The collected crude extract was kept in a tightly closed container till use. This was further tested for the presence of phytochemicals such as alkaloids, flavonoids, saponins and glycosides, etc.

Isolation of phytoconstituents from crude ethanolic extract

Collected ethanolic extract was treated with 60ml of 1N NaOH, following 9ml concentrated HCl. The precipitate obtained was further evaporated to dryness. The precipitate obtained after isolation procedure was tested for the presence of phytochemicals such as alkaloids, flavonoids, saponins and glycosides using the standard procedure.

Separation of isolated phytoconstituents

The obtained precipitate was subjected to thin layer chromatography. Optimized Mobile phase was selected for column chromatography i.e. Pet ether: ethyl acetate (8:2) for the purpose of separation of Phyto constituents. Two fractions were collected from the column.

Characterization of isolated fractions and Identification of phyto-compounds

The collected fractions were subjected to characterization using ultraviolet-visible spectroscopy, high-performance liquid chromatography, high-resolution spectroscopy, and gas chromatography-mass spectroscopy. Interpretation on mass-spectrum GC-MS and HRMS were conducted using the database of National Institute Standard and Technology (NIST), which archives more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

In vivo animal study

All animals were divided into five groups each containing six rats, and the selected dose was given to each animal (Table 1).

Induction of thrombocytopenia

Before inducing thrombocytopenia, values such as platelet count, bleeding time, and clotting time were determined on day 1. Thrombocytopenia in Group 2, 3, 4, 5 rats was induced by oral administration of Hydroxyurea at a dose of 1.6mg/kg for 24hrs. On the 2nd day, all animals were investigated for the development of thrombocytopenia.

Table 1 Study design for platelet augmentation of CPE and isolated phyto constituents

Groups	Treated with	Dose	No of animals
Group 1	Normal group	---	6
Group 2	Control group	1.6mg/kg	6
Group 3	Hyu induced group treated with Ethanolic Extract	200mg/kg	6
Group 4	Hyu induced group treated with fraction no 1	100mg/kg	6
Group 5	Hyu induced group treated with fraction no 2	100mg/kg	6

Treatment with crude ethanoilc extract, isolated phytoconstituents

A stock solution of ethanolic extract, Isolated Phytoconstituents were prepared by dissolving in distilled water. Based on body weight, the dose was administered orally using an oral gauge needle. The dose and volume of these phytoconstituents were calculated as per body weight. Blood samples were collected after 4hrs for 2days of treatment for platelet count determination and hematological parameter determination.

Determination of platelet count, RBC count, WBC count, Hb count, Hct count

Blood samples were collected from 4 hrs treatment through retro-orbital region in K3 EDTA tubes for platelet count determination.[16] Platelet count was determined by using a Haemocytometer.

Statistical analysis

Results were represented as figures. Mean +/- Standard Error of Mean (SEM) were calculated and compared using ANOVA. One-Way ANOVA with Dunnett's post-tests was performed to compare the control group to treated groups. Significance at $P<0.05$ was used for all comparisons. Statistical treatment of data was conducted using graph pad instat software.

Results and discussion

Powder characteristics of papaya leaf powder

By performing all powder characteristics it was found that papaya leaf powder was pure for further use, it was not overly contaminated with sand, dust, etc. (Table 2).

Table 2 Powder characteristics of papaya leave powder

Sr no.	Particulars	Observed value	Standard
1	Total ash	3.49	3.45-5.35
2	Acid-insoluble ash	1.12	----
3	Water soluble ash	2.01	----
4	Foreign Matter	4.34%	----
5	Loss on drying	8.40%	6.05-11.95

Phytochemical screening

Phytochemical screening of ethanolic extract and isolated Phytoconstituents shows the presence of Phytoconstituents as shown in Table 3.

Table 3 Phytochemical Screening

Tests For	Ethanolic extract	Isolated phytoconstituents
Alkaloids	+	-
Flavonoids	+	+
Glycosides	+	+
Tannins	-	-
Amino Acids	+	+
Proteins	+	-
Saponins	-	-

TLC of isolated phytoconstituents

TLC of isolated Phytoconstituents was performed on readymade TLC plates. Mobile phase was optimized i.e pet ethet: ethyl acetate (8:2) which was used to separate these compounds by column chromatography.

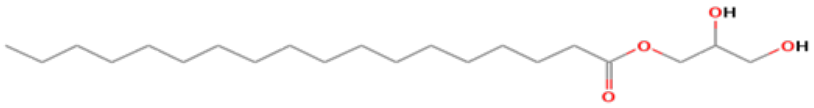
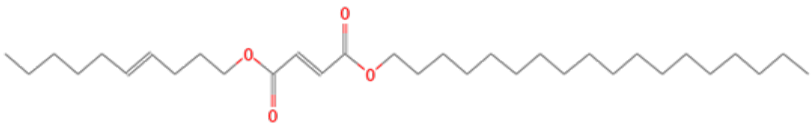
Column chromatography

Column chromatography of isolated constituents gives two separated bands which collected separately. These collected fractions were evaporated to dryness at room temperature.

HRMS analysis of fraction one

In the present study, HRMS analysis of fraction one was carried out at the central instrumentation facility of Savitribai Phule Pune University. The possible functional groups were analyzed by matching the m/z ratio with the already reported compounds in the NIST chemistry web book. By matching m/z ratio, probable presence of compounds was shown below Table 4. From the characterization of fraction one, it shows the presence of octadecanoic acid, 2,3-dihydroxypropyl ester, fumaric acid, dec-4-enyl octadecyl ester.

Table 4 m/z ratio and probable compounds

m/z ratio	Probable structure
358.3142	 Octadecanoic acid, 2,3-dihydroxypropyl ester
506.42	 Fumaric acid, dec-4-enyl octadecyl ester
688.58	Not identified
780.55	Not identified
962.72	Not identified

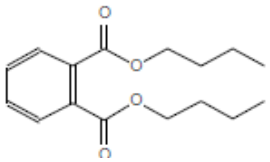
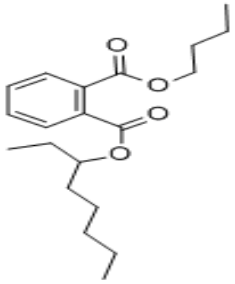
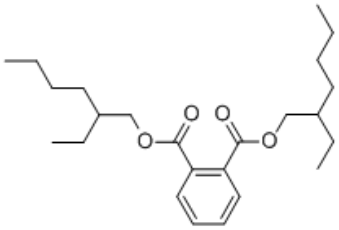
GCMS analysis of fraction two

In the present study, GC-MS profile of the fraction two from Carica papaya leaf ethanolic extract has shown 6 peaks representing phyto-compounds in the extract. The identified compounds were isobutyl phthalate, and phthalic acid esters. Retention time, area, and height were mentioned below (Table 5). The phyto-components in the ethanol extracts of the Carica papaya leaf were identified based on the retention time on DB 5-MS capillary standard non– polar column. Mass spectrum were interpreted using the database of National Institute Standard and Technology (NIST). The name, molecular weight, and structure of the components of the ethanol extract were identified and reported in (Table 6). Characterization of fraction two showed that this fraction was a mixture of some compounds and showed the presence of phthalic acid derivatives.

Table 5 Retention time, area (as digital units), peak area%, height and height%

Peak	Rt time	Area	Area%	Height	Height%
1	19.004	92211	2.58	51177	2.38
2	19.596	494525	13.83	242026	15.98
3	19.865	388700	10.87	149681	9.88
4	20.15	136029	3.81	66272	4.38
5	20.92	878898	2.46	41607	2.75
6	26.7	2375118	66.45	963988	63.64
		3574481	100	1514751	100

Table 6 Phyto-components identified in GC-Ms of fraction two

Line no.	Rt	Mol. weight	Formula	Probable compound
1	19.595	278	C16H22O4	1,2- Benzenedicarboxylic acid, dibutyl ester 
2	19.86	334	C20H30O4	Phthalic acid, butyl 2-Ethylhexyl ester 
3	20.16	306	Not found	Not found
4	26.7	390	C24H38O4	Phthalic acid, bis(2-ethylhexyl) ester 

Animal study

Feed and water consumption

The amount of feed and water consumed was measured daily from the quantity of feed and water supplied. This was measured from the start of the experimentuntil the end of the experiment. Results are expressed as mean±SEM. Comparison between the groups was made by one-way analysis of variance (ANNOVA) followed by Dunnett test. *, P<0.05, **P<0.01, ***P<0.001*= control compared to treated group. Hydroxyurea causes a decrease in feed and water consumption. After thrombocytopenia induction by using hydroxyurea (1.6mg/kg BW), feed and water consumption was found to decrease significantly. This effect was attenuated by treatment of all test samples. Among all test samples, fraction no.2 was found to be highly significant.

Platelet count

Figure 1 shows graphical presentation of platelet count. Results are expressed as mean±SEM. Comparison between the groups was made by one-way analysis of variance(ANNOVA) followed by Dunnett test *,P<0.05, **P<0.01,*** P<0.001. *=control compared to treated group Hydroxyurea causes a decrease in platelet count. After thrombocytopenia induction by using hydroxyurea (1.6mg/kg BW), platelets were found to decrease significantly. This effect was attenuated by treatment of all test samples. Among all test samples, fraction no.2 was found to be highly significant. Platelet count analysis was shown in Table 7.

Table 7 Platelet count analysis

Groups	Platelet count(per cu.mm)					
	Before thrombocytopenia induction	After thrombocytopenia induction	After treatment 4hr	12hr	24 hr	48hrs
Normal gr	6,32,000±2.345	6,28,000±3.42	6,29,000	6,30,000	6,27,000	6,29,000±1.78
Control gr	6,12,000±2.87	527000±2.654	515000	509000	575000	567000±0.998
Ethanolic extract	6,87,000±1.987	6,47,000±2.543	6,53,000	6,65,000	6,80,000	6,95,000±1.764*
Fraction 1	5,52,000±1.765	5,30,000±1.890	5,45,000	5,67,000	5,72,000	5,85,000±1.34
Fraction 2	9,12,000±1.654	8,84,000±1.654	9,60,000	9,95,000	10,15,000	10,36,000±1.766**

Mean±SEM, Dunnett test *,P<0.05, **P<0.01,*** P<0.001. *=control

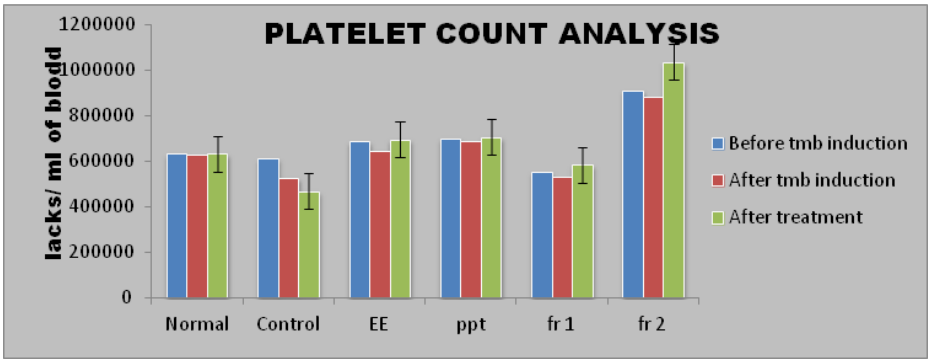


Figure 1 Platelet Count Graphical Presentation.

Conclusion

Thrombocytopenia is one of the major disorders which lead to death when no treatment is given. Hydroxyurea temporarily stops cells from dividing, especially the cells that divide rapidly. Red blood cells, white blood cells, and platelets are rapidly dividing cells and are made by the bone marrow. The need this paper aims to address is the discovery of a natural compound which is easily available, widely affordable, and has a direct bone marrow stimulating effect. In this regard, this study has been conducted so far using cytotoxic drugs to induce thrombocytopenia. A 3day study conducted with C.papaya leaf ethanolic extract, (200mg/ kg BW) isolated phytoconstituents and column fractions at a concentration of 100mg/kg in the hydroxyurea-induced thrombocytopenic rat. Treatment with ethanolic extract of Carica papaya leaf, isolated phytoconstituents and column fractions i.e fraction one (carboxylic acid ester derivative) and fraction two (phthalic acid ester derivative) produced a time-dependent increase in platelet count, red blood cell, white blood cell count and hematocrit which is significant ($p<0.05$) on 48hrs with respect to normal controls. Among the entire test samples, fraction two i.e. phthalic acid derivatives showed more significant results.

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Conflict of interest

Author declares that there is no conflict of interest.

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