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Antithrombocytopenic activity of carpaine and alkaloidal extract of *Carica papaya* Linn. leaves in busulfan induced thrombocytopenic Wistar rats

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ABSTRACT

Ethnopharmacological relavance: The decoction of *Carica papaya* Linn. leaves is used in folklore medicine in certain parts of Malaysia and Indonesia for the treatment of different types of thrombocytopenia associated with diseases and drugs. There are several scientific studies carried out on humans and animal models to confirm the efficacy of decoction of papaya leave for the treatment of disease induced and drug induced thrombocytopenia, however very little is known about the bio-active compounds responsible for the observed activity. The aim of present study was to identify the active phytochemical component of *Carica papaya* Linn. leaves decoction responsible for anti-thrombocytopenic activity in busulfan-induced thrombocytopenic rats.

Materials and methods: Antithrombocytopenic activity was assessed on busulfan induced thrombocytopenic Wistar rats. The antithrombocytopenic activity of different bio-guided fractions was evaluated by monitoring blood platelet count. Bioactive compound carpaine was isolated and purified by chromatographic methods and confirmed by spectroscopic methods (LC-MS and 1D/2D-1H/13C NMR) and the structure was confirmed by single crystal X-ray diffraction. Quantification of carpaine was carried out by LC–MS/MS equipped with XTerra® MS C_{18} column and ESI-MS detector using 90:10 CH_3CN:CH_3COONH_4 (6 mM) under isocratic conditions and detected with multiple reaction monitoring (MRM) in positive ion mode.

Results: Two different phytochemical groups were isolated from decoction of *Carica papaya* leaves: phenolics, and alkaloids. Out of these, only alkaloid fraction showed good biological activity. Carpaine was isolated from the alkaloid fraction and exhibited potent activity in sustaining platelet counts upto $555.50 \pm 85.17 \times 10^9$/L with no acute toxicity.

Conclusions: This study scientifically validates the popular usage of decoction of *Carica papaya* leaves and it also proves that alkaloids particularly carpaine present in the leaves to be responsible for the antithrombocytopenic activity.

1. Introduction

Thrombocytopenia is one of the disease associated with blood platelets, which occurs due to fall in platelet count below 20,000/μL (Brass, 2010; Thijs et al., 2010). The occurrence of thrombocytopenia is due to inhibition/improper platelet production and destruction of platelets by different means related to diseases induced (e.g. dengue, malaria), drug induced (chemotherapeutics) and other cause of thrombocytopenia (e.g. snake bite, niacin toxicity, lyme disease) (Gauer and Braun, 2012; Stasi, 2012).

Normally the first line of treatment includes use of corticosteroids, immunoglobulins and splenectomy, however it is not

Abbreviations: ITP, Idiopathic Thrombocytopenic Purpura; CPL, *Carica papaya* Linn. Leaves; MRM, Multiple Reaction Monitoring; GLP, Good Laboratory Practice; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; IS, Internal Standard; LLOD, Lower Limit of Detection; LLOQ, Lower Limit of Quantitation; % RE, Percentage Relative Error; % CV, Percentage Coefficient of Variation; SEM, Standard Error of Mean; ICH, International Conference on Harmonization; API, Atmospheric Pressure Ionization

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effective for 25–30% of the patients with chronic Idiopathic Thrombocytopenic Purpura (ITP) (McMillan, 1997).

Carica papaya belongs to Caricaceae family, is a perennial, soft wooded, economically important plant lives about 5–10 years, cultivated globally for their fruit (Duke, 1983; Silva et al., 2007). Preliminary phytochemical screening discovered the presence of carbohydrates, amino acids, saponin, glycosides, iridoids, flavonoids, phenolics, and alkaloids (Zunjar et al., 2011). The health benefit of all parts of papaya is well known and considered worldwide (Krishna et al., 2008).

C. papaya leaves suspension in palm oil was administered for the treatment of dengue in Swiss albino mice and observed elevation in blood platelet counts (Sathasivam et al., 2009). However, Sharma and Mishra (2014) and Manohar (2013) have raised concern related with scientific evidences for the use of *C. papaya* leaves extract to increase the blood platelet count in dengue. Whereas, increase in platelet count in patients that were suffering from dengue after oral administration of papaya leaves extract has been observed (Siddique et al., 2014; Arya and Agarwal, 2014). Similarly, Yunita et al. (2012) have studied the effect of *C. papaya* leaf capsules on patient suffering from dengue fever. The results showed significant increase in blood platelet count up to optimum level. Comparable results have also been reported by Subenthiran et al. (2013) in Malaysia where the effects of *C. papaya* leaves on 228 dengue patients were studied. Gene expression tests were also carried out by the same group on the ALOX 12 and PTAFR genes and found to be highly expressed in the patients treated with papaya leaves. Patil et al. (2013) have reported a good antithrombocytopenic activity with infusion of *C. papaya* leaf extract on the thrombocytopenic model of Wistar rat induced by cyclophosphamide. Prevention of fall in blood platelet count in myelosuppressed Swiss albino mice induced by carboplatin was found to be done with the help of papaya leaf juice (Tahir et al., 2014). Management of thrombocytopenia in Sri Lanka is being done using mature leaf concentrate of *C. papaya* as traditional medicine which was scientifically validated on adult Wistar rat model induced by hydroxyurea (Gammulle et al., 2012).

Hematological, biochemical parameters and toxicological changes in a murine model were studied by Dharmarathna group and revealed potential and significant role of fresh *C. papaya* leaf extract (Dharmarathna et al., 2013).

The antiplasmodial activity of papaya leaves has been studied by Julianti et al. in *in vitro* and in *Plasmodium berghei* mouse model. For the identification of the active compounds responsible for the antiplasmodial activity, HPLC based activity profiling was used and on the basis of *in vitro* studies the active compounds were identified as the piperidine alkaloid. They have also found carpaine to be the most effective fraction for antiplasmodial activity. However the same activity was not observed in *in vivo* murine model (Julianti et al., 2014).

Aziz et al. (2015) have reported significant increase in thrombocytopoietic cytokines (IL-6 and SCF) in *in vitro* culture of peripheral blood leukocytes and stem cells from human exfoliated deciduous teeth using unripe, peeled fruit pulp juice of *C. papaya*.

Otsuki et al. have studied the effect of decoction of *C. papaya* leaf extract on the proliferative responses of tumor cell lines and human peripheral blood mononuclear cells (PBMC), and assessed cytotoxic activities of PBMC by [3H]-thymidine incorporation using different techniques like flow cytometry, ELISA, microarray analysis and RT-PCR. The results suggested that the papaya leaf extract may potentially provide the means for the treatment and prevention of selected human diseases such as cancer, various allergic disorders, and may also serve as immune-adjuvant for vaccine therapy (Otsuki et al., 2010).

The identification of carpaine as active component for antiplasmodial activity has been reported (Julianti et al., 2014) however, so far identification of active component responsible for antithrombocytopenic activity from the *C. papaya* has not been done. So in this study, an attempt was made to evaluate the antithrombocytopenic activity of *C. papaya* plant and to identify the chemical constituent present therein responsible for the biological activity. The pharmacological study was carried out on chemically induced thrombocytopenic model in Wistar rats. Bioassay guided fractionation was performed to identify the active phytochemical group. The chief chemical constituent responsible for the observed activity was identified, characterized and quantified.

2. Materials and methods

2.1. 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). Ferric chloride, bismuth nitrate, sodium sulfate and busulfan were purchased from Sigma Aldrich (India). Polyethylene glycol 400 and silica gel 60F 254 plates were purchased from Merck (Darmstadt, Germany). Agar was obtained from HiMedia Lab (India) and Whatman no. 1 chromatographic papers were used as received. All solvents used for LCMS analysis were of LCMS grade and purchased from Sigma Aldrich (India).

2.2. Plant material

C. papaya Linn., Family Caricaceae, matured healthy leaves of male and female plants of *C. papaya* were collected from Bamangaon, Vadodara, Gujarat, India in December 2012. The plant was taxonomically classified by the botanist Prof. M. Daniel from the Department of Botany, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. Voucher specimens were deposited in the Herbarium of the same Institute for future reference (No. BARO/2010/51). Mature leaves of male and female plant of *C. papaya* were thoroughly washed with distilled water to remove dust. Petiole and veins were removed. Remaining portion was shade dried for a day followed by complete drying in an oven at 38 °C, grounded using a mortar and pestle for 10–15 min and the powder was preserved under nitrogen.

2.3. Bio-guided fractionation

Twenty percent w/v of the dry leaves powder was boiled in distilled water for 60 min in the preparation of decoction (PNWE). Decoction was initially filtered through muslin cloth followed by Whatman no. 1 filter paper. The filtrate was lyophilized and preserved in an air tight container.

PNWE were digested in 90% ethanol and 1% acetic acid for 48 h followed by filtration and concentration of the filtrate under *vaccuo*. The concentrate obtained was repeatedly washed with ether to remove chlorophyll. The aqueous fraction was alkalinated with ammonia and kept at 0–5 °C for 24 h in dark. The liberated base was extracted with ether and evaporated to give crude alkaloids (PNAL). The alkaloids were obtained as a sticky light brown solid and tested against Dragendorff's reagent.

The PNAL extract was further fractionated into 2 different fractions *viz*. petroleum ether extract and ethyl acetate extract. As the name suggests, they were extracted in the respective solvents with different polarity (petroleum ether with 0.1 and ethyl acetate with 4.2 polarity index). Carpaine was isolated from the alkaloid fraction (PNAL) by extraction in n-hexane, followed by recrystallization from acetone (Govindachari et al., 1954). Structural