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SHORT COMMUNICATION article

Effect of *Carica papaya* Linn (Caricaceae) leaf extracts on reactive oxygen species production

Laila M. Abdelrahim [*], and **Hanan F. Hiblo**

Department of Oral Maxillofacial Clinical Science, Faculty of Dentistry, Alasmarya University, Zliten, Libya
[*] Author to whom correspondence should be addressed

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Keywords: *Carica papaya* leaf, reactive oxygen species, ROS, production, platelet count

Abstract: *Carica papaya* leaves are a popular remedy to treat Dengue-induced thrombocytopenia. There have been reports of *Carica papaya* effects as antibacterial, anti-parasitic, anti-cancer, and anti-inflammatory. There is limited evidence documenting the effects of *Carica papaya* on increasing platelet count in relation to Dengue infection. Moreover, the mechanism by which *Carica papaya* increases the platelet counts has not been elucidated. Understanding how *Carica papaya* affects platelet function may have implications beyond Dengue fever management. This study aims to investigate the mechanism by which *Carica papaya* affects platelet count. The mechanism of thrombocytopenia in the aqueous and methanol extracts was elucidated by measuring reactive oxygen species in platelets using chemiluminescence. Both aqueous and methanol extracts of *Carica papaya* leaves were found to increase reactive oxygen species levels *in vitro*. This study is the first to try to demonstrate the mechanism by which *Carica papaya* increases platelet count. *Carica papaya* leaves may be developed into novel and useful therapeutic agents in the management of thrombocytopenia-related disorders and cardiovascular diseases.

Introduction

Currently, modern sciences accept the use of herbs as a source of new bioactive compounds [1]. Plants are a rich source of potential leads for drug discovery against various diseases [2-4]. The recent development of the science of phytopharmaceuticals has generated renewed enthusiasm for herbal drug research, leading to the discovery of new medicines [5, 6]. Globally, *Carica papaya* is being cultivated widely as a fresh, dried, or crystallized fruit as well as for use in drinks, jams, and candies. In Southeast Asia, the green fruit, leaves, and flowers, as well as the young shoots, can be consumed as a vegetable [7]. Several studies have been conducted to evaluate the biological activities of the different parts of the plant, which include the fruits, shoots, leaves, rinds, seeds, roots, and latex [2, 4, 8]. Apart from food, the *Carica papaya* plant has also been used for medicine by various communities around the world. Gill reported that different parts of *Carica papaya* have been used in folk medicine [9]. According to Ayoola and Adeyeye [10], *Carica papaya* leaf (CPL) are considered to possess some nutritional value as they were found to contain multi-nutrients, in varying proportions (carbohydrates: 8.3%, ascorbic acid: 38.6%, protein: 05.6%, minerals like magnesium: 0.035%, iron: 0.0064%, and phosphoric acid: 0.225% per 100 gm of edible portion) [11]. These components are considered to be responsible for the role of the leaves in the proper functioning of the heart, coagulation of

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blood and nervous system, normal contraction of muscles, water metabolism, promotion of digestion, assimilation, osmosis, proper functioning of the pituitary gland, the pineal gland and the brain, promoting hepato-renal function, combating anemia, and helping in normal growth [12]. CPL in particular has been used in the treatment of dengue fever, particularly in South East Asia [13]. One of the complications often associated with Dengue fever is thrombocytopenia. Therefore, it is reasonable to suspect that CPL may influence platelet formation or its activity. Thus, understanding this effect may lead to real potential for CPL to be developed into useful pharmacological agents affecting platelet production and function important in the management of diseases affecting platelets, like Dengue fever, or those affected by platelet function, like cardiovascular diseases. Therefore, this study aims to determine the mechanism of action and the thrombocytopoietic effects of CPL aqueous and methanol extracts. Conventional synthetic pharmacological agents are generally costly, resulting in a significant burden on developing countries' healthcare budgets. Developing treatment from natural sources will provide a more cost-effective alternative, as most developing countries lie within or near the equatorial belt, which is a suitable area for *Carica papaya* plantation.

Materials and methods

Plant extract preparation: Carica papaya extract was prepared from powdered leaves using two solvents, namely water and methanol.

Preparation of the aqueous extract: The whole leaves of dried and powdered CPL were supplied from the USA (the origin of CPL is from India). 200 grams of powdered leaves were dissolved in 2000 ml of distilled water and heated at 70˚C for 60 minutes. The extract was subsequently filtered through Whatman filter paper no. 1 using a funnel. The filtrate was collected and further heated at 60˚C-70˚C to reduce the volume to less than half (~600 ml). The concentrated extract was then dispensed into individual aliquots and dried in the oven at 40˚C-50˚C for three days. The completely dried extract was then scraped and collected. A final derived extract (10.0 g) was stored at room temperature for further use [14].

Preparation of the methanol extract: 600 grams of powdered leaves were soaked in 6000 ml of methanol for 24 hours. Later, the extract was filtered through the Whatman filter paper no. 1. The filtrate was subsequently concentrated using a rotary evaporator maintained at 55˚C. Finally, the concentrated extract was dispensed into individual aliquots and dried at 65˚C for 10 days until completely dried. A final collected methanol extract (30.0 g) was stored at 04˚C for subsequent use.

Study subjects and blood samples: A pool of healthy volunteers has been established for the *in vitro* study. A total of 43 male or female volunteers aged 18 to 45 years have been screened. For each experiment, the blood sample is sourced from four different individual volunteers. The selection criteria for healthy volunteers include those without any long-term medical therapy and abstaining from any drugs known to affect platelet count and function in the last two weeks before participation in the study. No history of any cardiovascular-related disease (stroke, hypertension), non-smoker, no known allergy to medicines, and abstained from any drugs known to affect platelets' function and numbers in the previous two weeks before the study. Menstruating women, pregnant women, and participants who had a phlebotomy procedure (blood donation or blood taking) were excluded from the study due to the possibility of unreliable results or platelet non-responsiveness. A total of 30.0 ml of blood was extracted from each subject. An honorarium was given to the study participants after obtaining the blood specimens. Blood was collected by venipuncture and immediately placed into 15.0 ml polypropylene tubes (Greiner Bio-one, Germany) containing anticoagulant 03.2% sodium citrate with 9 to 1 ratio of blood and anticoagulant for centrifugation. Human platelets were obtained from whole blood, and collection, transport, and centrifugation steps were performed at room temperature.

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Determination of platelets induced reactive oxygen species (ROS) production: Washed platelets (WP) were prepared using the method described by Baenziger and Majerus [15]. Briefly, human blood from the volunteers was mixed with 03.2% (w/v) sodium citrate solution, which was then centrifuged at 3000×g for 15 min. Platelet-rich plasma (PRP) was further centrifuged at 6000×g for two minutes. The pellets were then re-suspended with HEPES Tyrode's buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 2.68 mmol/l KCl, 0.42 mmol/l NaH2PO4, 1.7 mmol/l MgCl2, 11.9 mmol/l NaHCO3, 5.0 mmol/l glucose). WP were incubated with 1.0 mg/ml CPL extracts for 30 min. O^{2-} production was then measured by a chemiluminescence assay using dye L-012, which has high specificity and sensitivity. The reaction solution consists of 100 µL calcium chloride (1.0 mM) and 100 µL L-012 (100 µM), which was added to 100.0 µL of either CPL aqueous extracts or methanol-treated WP. Finally, collagen in 10-30 µg/mL or adenosine diphosphate, ADP, in 100-300 µM was added to the samples. Photon emission was expressed as the percent increase in relative light unit versus control conditions [16].

Ethical approval: Informed consent was obtained from all subjects before enrolment in the study. The volunteers also signed the consent form for future blood taking.

Statistical analysis: The parameters were analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, USA) software and were expressed as mean±S.E.M. Differences in the continuous variables between the study groups and control were analyzed using Student's *t*-test or one-way Analysis of Variance (ANOVA), followed by Tukey's pairwise comparison, as appropriate. A $P < 0.05$ was considered statistically significant.

Results

Effect of CPL extracts on ROS production: To determine the production of ROS by CPL extracts *in vitro*, human WP were used in this experiment. Both ADP and collagen-stimulated WP demonstrated an increase in ROS production as compared to the unstimulated WP. In ADP-stimulated WP, both CPL extracts were found to increase ROS production. For instance, CPL aqueous and methanol extract increases ROS production to 151.7±8.6% and 131.4±9.0%, respectively, as compared to 124.9±12.2% in the unstimulated control (**Figure 1**). Similar observations are observed in collagen-induced WP. CPL aqueous and methanol extract increases the ROS production to 125.3±6.0% and 149.6±7.3%, respectively, as compared to 115.0±8.3% in the unstimulated control (**Figure 2**).

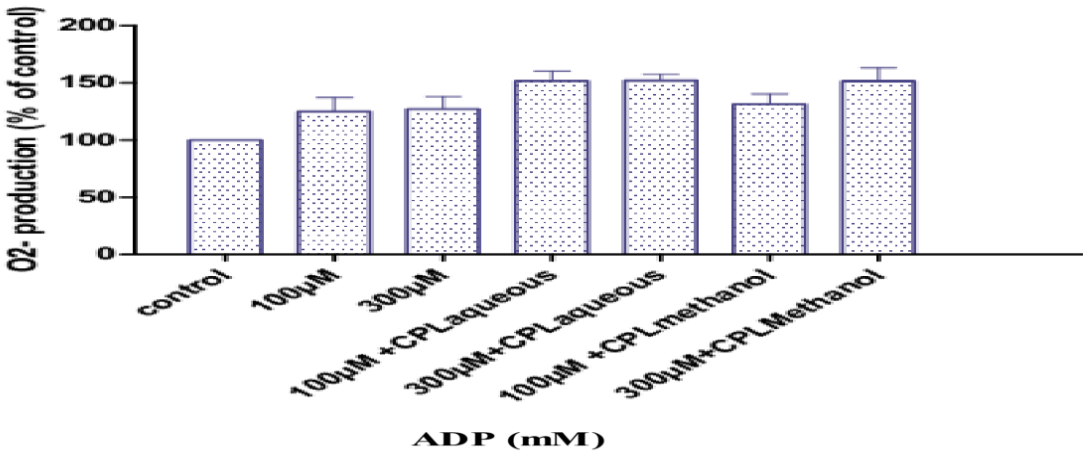

Figure 1: CPL aqueous and methanol extracts induce intracellular ROS production in platelets. WP were preincubated with both extracts for 30 min at 37°C. Platelets were then stimulated with ADP at 100 µM and 300 µM, followed by measurement of intracellular ROS production. ROS production was induced by aqueous and methanol extracts of CPL as compared to the control. Production of platelets O^{2-} was expressed as a percentage. One-way ANOVA was performed, and the means between the treated and control groups were not significant. Data were expressed as mean ±S.E.M., n=4.

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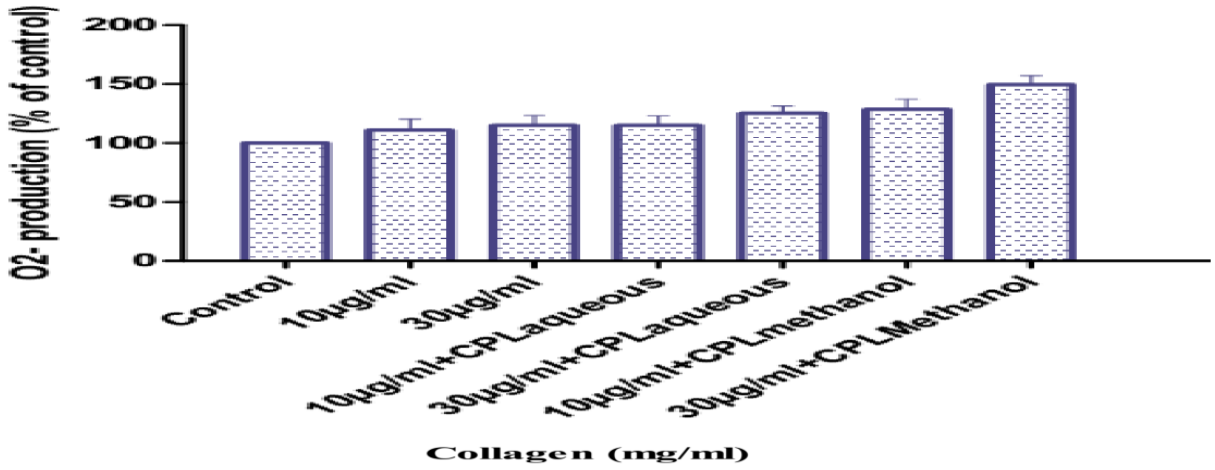

Figure 2: CPL aqueous and methanol extracts induce intracellular ROS production in platelets. WP were preincubated with both extracts for 30 min at 37°C. Platelets were then stimulated with collagen (10 µg/mL and 30 µg/ml), followed by measurement of intracellular ROS production. ROS production was induced by aqueous and methanol extracts of CPL as compared to the control. Production of platelets O^{2-} was expressed as a percentage. One-way ANOVA was performed, and the means between the treated and control groups were not significant. Data were expressed as mean±S.E.M., n=4.

Discussion

The current has determined an increase in ROS production *in vitro* by CPL extracts in human platelets. This suggested that ROS formation stimulated by CPL extracts may lead to the fragmentation and differentiation of MKs, thus increasing platelet count [17-19]. Platelet production is a chronological process beginning with hematopoietic stem cell proliferation and ending with the fragmentation of MKs. ROS have been identified as an important mediator of cell proliferation and differentiation [20]. ROS includes superoxide, hydrogen peroxide, and peroxynitrite, which are generated by all cells of the body. ROS promotes cell signaling pathways and is modulated by either growth factors or transcription factors; therefore, it regulates cell proliferation, differentiation, and apoptosis [21]. Exposure to low concentrations of hydrogen peroxide and/or superoxide anion stimulates the growth of a variety of cell types, among them smooth muscle cells, fibroblasts, prostate cancer cells, and aortic endothelial cells [20]. ROS affects the fate of hematopoietic stem cells by regulating the ROS-phosphatidylinositol-3-kinase (PI3K)/Akt-transcription factor forkhead box O (FOXO)-3 and ROS-p38 mitogen-activated protein kinase-a (MAPKa) pathways. Platelet production is a result of MK apoptosis, which may be caused by elevated levels of ROS.

Conclusion: The thrombocytopoietic effects of *Carica papaya* leaf may have been mediated by the stimulation of reactive oxygen species formation. This knowledge may help the development of *Carica papaya* leaf into a useful therapeutic agent for the management of thrombocytopenic-related disorders.

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Data availability statement: The raw data that support the findings of this article are available from the corresponding author upon reasonable request.
Author declarations: The authors confirm that they have followed all relevant ethical guidelines and obtained any necessary IRB and/or ethics committee approvals.