ORIGINAL ARTICLE

Modulation of platelet functions by *Careya sphaerica* Roxb. leave extracts

Warachate Khobjai, Wanvisa Ninlaor[1], Worawan Watcharasamphankul[2], Thaksaorn Thongom[1], Suriyan Sukati[3]

Department of Preclinic, Faculty of Medicine, Siam University, Phasi Charoen, Bangkok, [1]Department of Thai Traditional Medicine, Thai Traditional Medicine College, Rajamangala University of Technology Thanyaburi, Pathum Thani, [2]Department of Clinical Microbiology, Faculty of Medical Technology, Nation University, Lampang, [3]Department of Medical Technology, School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat, Thailand

J. Adv. Pharm. Technol. Res.

ABSTRACT

Platelets form a plug to prevent blood loss and contribute to wound healing. Kradonbok, *Careya sphaerica* Roxb., is a Thai plant with medicinal properties. Conventionally, leaves of *C. sphaerica* are being used to help wound healing in Thailand. The present study was aimed to investigate the effect of *C. sphaerica* on the function of platelet. Four different extracts of leaves of *C. sphaerica* (distilled water, methanol, ethanol, and chloroform extracts) were prepared. The extracts at 5.0 mg/ml per dose were tested for the effect of *C. sphaerica* on platelet adhesion and aggregation properties, by employing a microtiter plate approach. The phytochemical identification was done by using gas chromatography–mass spectrometry (GC-MS). Our data revealed that chloroform extract significantly activated thrombin-induced platelet adhesion (105.27 ± 0.11%, $P < 0.05$). None of the extracts exhibited an improvement in platelet aggregation. Further GC-MS analysis of the chloroform extract revealed five key phytochemical constituents with potential platelet activation properties. In conclusion, our study evaluated platelet activation and potentially wound healing property of *C. sphaerica*. GC-MS analysis identified potential bioactive phytochemical compounds in *C. sphaerica* which warrant further investigation to characterize these compounds.

Key words: *Careya sphaerica* roxb, platelet adhesion, platelet aggregation, primary hemostasis, wound healing

INTRODUCTION

Hemostasis is an essential physiological process to stop bleeding and help wound healing. When an injury occurs, platelets play an essential role in primary hemostasis and the secondary hemostatic process of coagulation-comprising intrinsic and extrinsic pathways.[1] In addition, platelets are involved in thrombin generation.[2] Hence, platelets promote both processes of primary and secondary hemostasis. The mechanical properties of a blood clot are essential for proper hemostasis and wound healing.[3] Blood clot formation depends on several factors, most notably the structure of the fibrin polymer as well as how permeable it is, which, in turn, affects accessibility to incoming repair cells.[3]

A primary hemostatic plug is formed by aggregation of platelet whereby fibrinogen binding to platelets constitutes an important part of wound healing.[3] One of the important mechanisms of platelet function is adhesion to the damaged vessel wall, which is considered an essential process of platelet aggregation.[4] When platelets adhere to collagen

Address for correspondence:
Dr. Suriyan Sukati,
Department of Medical Technology, School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat, 80160, Thailand.
E-mail: suriyan.su@wu.ac.th

Submitted: 13-Apr-2021 **Revised:** 29-Jun-2021
Accepted: 19-Jul-2021 **Published:** 20-Oct-2021

Access this article online	
Quick Response Code:	Website: www.japtr.org
	DOI: 10.4103/japtr.japtr_95_21

How to cite this article: Khobjai W, Ninlaor W, Watcharasamphankul W, Thongom T, Sukati S. Modulation of platelet functions by *Careya sphaerica* Roxb. leave extracts. J Adv Pharm Technol Res 2021;12:420-4.

at the subendothelium of vessel, they begin platelet aggregation processes. Therefore, analysis of the adhesion and aggregation functions of platelets is of great importance in the differential diagnosis and follow-up of bleeding and thrombotic syndromes.

In Thailand, many native plants are used for food and traditional medicine. *Careya sphaerica* Roxb, called Kradonbok in the Thai language, is an indigenous vegetable. It is commonly found in the Southern part of Thailand. Local people favorably consume the fresh young leaves and flowers. There is evidence that *C. sphaerica* leaves have health benefits such as accelerated wound healing.[5] Thus, this study aimed to determine the effects of *C. sphaerica* leaves on platelet activation.

MATERIALS AND METHODS

Plant extraction and preparation

C. sphaerica leaves were collected from three different trees in Na Yong District region, Trang Province, Southern Thailand, in May 2017. The collected plants were dried in a storage space at room temperature (RT). The plant specimens were identified by Walailak University (WU) Herbarium and the voucher specimens were deposited at WU Herbarium (voucher specimen number: WU1147-9), Walailak Botanic Garden, WU, Nakhon Si Thammarat, Thailand. The distilled water extraction was performed by decoction technique, using boiling with distilled water. The powder portion of *C. sphaerica* leaves (10% w/v) was soaked in boiling distilled water for 30 min at RT with occasional stirring. The solution was filtrated through Whatman's filter paper No. 1 and was then concentrated by lyophilization. Extraction by maceration in methanol, ethanol, and chloroform was performed at a ratio of 1:10% w/v, incubated at RT for 7 days. The precipitates were centrifuged at 2000 rpm and then evaporated by a rotary evaporator. To measure platelet activity, the platelet adhesion and platelet aggregation tests were employed.

Blood samples

Peripheral blood samples were collected from 30 healthy human volunteers. Volunteers had no history of oral contraceptive or anticoagulant therapy. The blood was placed separately in containers containing 3.2% sodium citrate. Centrifugation was carried out at 100×g for 10 min at 22°C, to separate the blood cells from plasma to harvest platelet-rich plasma (PRP). The PRP was employed for platelet adhesion and platelet aggregation tests. Study design and informed consent form for the volunteers were permitted by the Committee on Human Rights Related to Human Experimentation of Walailak University (reference number WUEC-18-024-01).

Platelet adhesion assay

The adhesion function of platelet was analyzed by a microtiter plate method using 96-well microtiter plates coated with 50 µg/ml collagen overnight.[6] The collagen solution was removed, the well washed three times with phosphate-buffered saline (PBS). Then, 25 µl of both PRP (2×10^8 cells/ml) and the sample were added to the well. After incubating for 10 min at RT, the mixtures were activated with 0.25 units/ml of thrombin. The reaction was then incubated for 30 min at RT. Absorbance at 600 nm was measured on Glomax Multi microplate reader, USA. Aspirin was used as the positive control and PBS as the negative control. The percentage of platelet adhesion was calculated by the formula following:

$$\% \text{ platelet adhesion} = As/Ac \times 100$$

Where As is the absorbance of the sample and PRP solution, and Ac is the absorbance of the PBS and PRP without sample extract.

Platelet aggregation assay

The aggregation of platelets was determined by a microplate method.[7] Common methods for evaluating aggregation are based on the decrease of turbidity of a platelet suspension. A modification of this method, consisting of the measurement of absorptivity at 600 nm in microtiter plates, allowed detection of platelet aggregation under conditions similar to those used for the adhesion assay. The 96-well microtiter plates were added with 25 µl of 2×10^8 cells/ml PRP and 25 µl of the sample. The reaction was incubated at RT for 10 min. Then, 25 µl of 50 µg/ml collagen was added into each well of the 96-well microtiter plate. Absorbance at 600 nm was recorded at 0 and 20 min incubation time. Aspirin was used as the positive control and PBS as the negative control. Platelet aggregation percentage was calculated by the formula following:

$$\% \text{ platelet aggregation} = As/Ac \times 100$$

Where As is platelet aggregation absorbance of sample and Ac is platelet aggregation absorbance of the PBS control.

Gas chromatography–mass spectrometry analysis

Gas chromatography–mass spectrometry (GC-MS) analysis was performed on an Agilent 7890, GC column HP-5MS, and interfaced to a 5975C inert mass selective detector with triple-axis detector. For the experiment, 2 µl of sample was injected using a 10:1 split ratio. The injection, outlet, and MS transfer line temperatures were set to 250°C, 280°C and, 250°C, respectively. The mass spectra were recorded at 70 eV and fragments from 40 to 700 Da were analyzed.[8] Identification of the constituents was conducted by comparing their mass spectra with fragment data from the National Institute of Standards and Technology (NIST).

Statistical analysis

All values are expressed as mean and standard deviation. Descriptive statistics and paired *t*-test were analyzed using GraphPad Prism 6 (version 6.01, GraphPad Software,

CA, USA). A value of $P < 0.05$ was indicated statistically significant.

RESULTS AND DISCUSSION

Platelet adhesion activity

Platelet adhesion activity was determined using the collagen-coated microtiter plate. The effect of *C. sphaerica* extracts with different solvents at 5.0 mg/ml on human platelets adhesion activity was determined [Figure 1]. The percentage of thrombin-stimulated platelet adhesion following the addition of distilled water, methanolic, ethanolic, and chloroform extracts was 61.98 ± 1.44, 65.23 ± 0.52, 67.4 ± 2.79, and 105.27 ± 0.11, respectively. When compared to the negative control, phosphate-buffered saline (PBS), the chloroform extract could significantly increase platelet adhesion ($P < 0.05$). However, the distilled water, methanolic, and ethanolic extracts significantly inhibit platelet adhesion ($P < 0.001$).

Platelet aggregation activity

The effect of the extracts with different solvents at 5.0 mg/ml on human platelet aggregation is presented in Figure 2. The percentage of collagen-stimulated platelet aggregation following the addition of distilled water, methanolic, ethanolic, and chloroform extracts was 46.84 ± 6.87, 52.95 ± 0.04, 96.40 ± 2.41, and 91.60 ± 3.92, respectively. No extracts could induce the aggregation of platelets when compared to control, but the distilled water and methanolic extracts surprisingly showed significant inhibition in the collagen-induced aggregation ($P < 0.001$).

Gas chromatography–mass spectrometry analysis

Phytochemical compounds of chloroform extract were characterized and identified using GC-MS. Retention time, name, molecular formula, molecular weight, and amount (% area) of compounds are shown in Table 1. The chromatogram of the *C. sphaerica* extract was compared with the mass spectra of the compounds of NIST version 11 library. Results of the GC-MS chromatogram analysis of *C. sphaerica* chloroform extract showed five main constituents, including clomesone, pyrido[2,3-d] pyrimidine, 4-phenyl-, 6-nitro-1H-quinazoline-2,4-dione, 2-chloroethyl thiocyanate, and 1H-indole, 1-methyl-2-phenyl.

Clomesone, the 2-chloroethyl ester of methanesulfonic acid, induces the formation of DNA interstrand crosslinks and exhibits antitumor activity.[9] 4-phenyl-pyrido[2,3-d] pyrimidine has a variety of pharmacological activities such as analgesic, antimicrobial, anti-allergic, antihypertensive, antitumor, anti-leishmanial, diuretic, anti-inflammatory, anti-tuberculostatic, anticonvulsant, potassium-sparing, and anti-aggressive properties.[10] 6-nitro-1H-quinazoline-2,4-dione is quinazoline derivatives

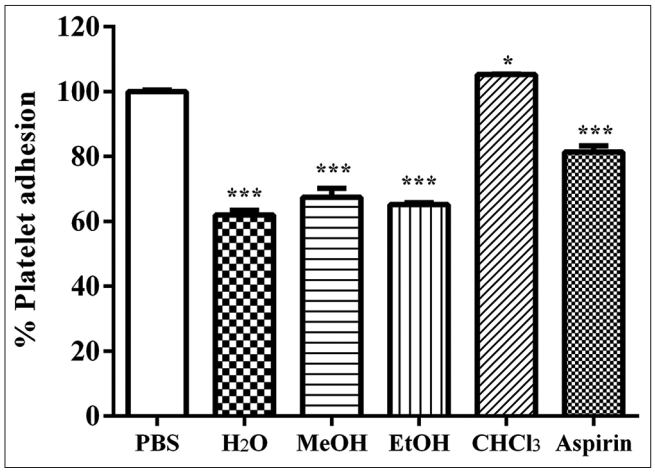

Figure 1: Platelet adhesion activities of different extracts of *Careya sphaerica*. *$P < 0.05$ and***$P < 0.001$, compared with phosphate-buffered saline

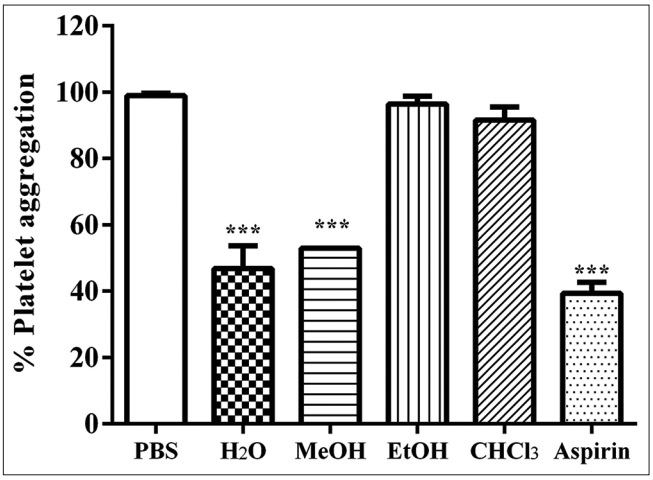

Figure 2: Platelet aggregation activities of different extracts of *Careya sphaerica*. ***$P < 0.001$, compared with phosphate-buffered saline

Table 1: Phytocomponents identified in chloroform extract of *Careya sphaerica* leaves by gas chromatography–mass spectrometry

RT	Compound	MF	MW	Percentage area
26.15	Clomesone	$C_4H_9ClO_5S_2$	236	0.58
26.17	Pyrido[2,3-d] pyrimidine, 4-phenyl-	$C_{13}H_9N_3$	207	1.36
26.35	6-Nitro-1H-quinazoline-2,4-dione	$C_8H_5N_3O_4$	207	3.35
26.38	2-Chloroethyl thiocyanate	C_3H_4ClNS	121	1.25
26.41	1H-Indole, 1-methyl-2-phenyl-	$C_{15}H_{13}N$	207	4.13

RT: Retention Time, MF: Molecular formula, MW: Molecular weight

which possess anti-inflammatory and antiviral properties.[11,12] 2-chloroethyl thiocyanate can be used for anti-inflammatory and antimicrobial action to boost the host's defense system while reducing tissue inflammation.[13] 1-methyl-2-phenyl-1H-indole is a novel compound used for dementia disorders treatment.[14] Indole and its derivatives show pharmacological activities such as virus inhibitors and peroxisome proliferator-activated receptor gamma activators.[15,16] We assume that bioactivities exhibited by *C. sphaerica* in this study are correlated to the existence of one or many of these phytochemical compounds.

Wound healing, a physiological reaction to tissue injury, comprises four main processes, including hemostasis, inflammatory phagocytic, proliferative fibroblastic, and maturation remodeling.[17] Platelets play a crucial role in hemostasis which is the process of the wound being closed by clotting to stop the loss of blood. Once platelets adhere to collagen at the subendothelium of the damaged vessel, platelets are induced to release adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2). Releasing of ADP from platelets results in platelet aggregation and initiates the process of clot formation.[18]

C. sphaerica is a Thai traditional medicine plant locally known as Kradonbok. Its leaf is used for wound healing. Therefore, it may be useful as a therapeutic agent targeted at the hemostatic process, especially platelet function. This study shows that only the chloroform extract of *C. sphaerica* extract could possibly activate platelet function through induction of platelet adhesion but not platelet aggregation. This observation indicates that nonpolar phytoconstituent(s) present in the chloroform extract may get involved in platelet adhesion induction. Although the chloroform extract could not activate platelet aggregation *per se*, induction of platelet adhesion to collagen could trigger the secretion of platelet granule substances leading to platelet aggregation. The activated platelets could play a crucial role in wound healing because they express many mediators, including multiple cytokines and vascular endothelial growth factors, that help to promote cell recruitment, tissue regeneration, and matrix remodeling.[19] In normal wound healing, the fibrinolytic pathway also plays a crucial role by removal of fibrin removal. Fibrin deposition is a feature of nonhealing wounds and may be an important pathogenic component that prolongs hemostasis.[20] The previous study has demonstrated that the methanolic extract from *C. sphaerica* acted as fibrinolytic enzymes.[21] Thus, its fibrinolytic property could contribute to wound healing as well. In contrast to the chloroform extract, the distilled water, methanolic, and ethanolic extracts have shown antiplatelet activity, whereas the highest inhibitory effect was observed with the extract in the distilled water. There are many phytoconstituents present in plants such as phenolics, carotenoids, and vitamins which inhibit platelet adhesion and aggregation.[22-24]

CONCLUSION

This study demonstrated that the chloroform extract of *C. sphearica* leaves could possibly stimulate platelet adhesion. Bio-active phytoconstituents from nonpolar extract of *C. sphearica* could potentially be used for wound healing. Isolation and characterization of the specific compound (s) in the extract demonstrating platelet adhesion activity should be further investigated. Moreover, the role of *C. sphearica* leaves in other wound healing processes, including inflammatory, proliferative, maturation, and remodeling phases, should be studied.

Acknowledgment

This study was supported by grant IRD60001 from Rajamangala University of Technology Thanyaburi, and grant WU60307 from Walailak University. We would like to thank Dr. Gerd Katzenmeier, School of Allied Health Sciences, Walailak University, for critical reading of the manuscript.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Sang Y, Roest M, de Laat B, de Groot PG, Huskens D. Interplay between platelets and coagulation. Blood Rev 2021;46:100733. [doi:10.1016/j.blre.2020.100733].
2. Hou Y, Carrim N, Wang Y, Gallant RC, Marshall A, Ni H. Platelets in hemostasis and thrombosis: Novel mechanisms of fibrinogen-independent platelet aggregation and fibronectin-mediated protein wave of hemostasis. J Biomed Res 2015;29:437-44. [doi:10.7555/JBR.29.20150121].
3. Golebiewska EM, Poole AW. Platelet secretion: From haemostasis to wound healing and beyond. Blood Rev 2015;29:153-62.
4. Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. Circ Res 2007;100:1673-85.
5. Maisuthisakul P, Pongsawatmanit R. Effect of sample preparation methods and extraction time on yield and antioxidant activity from Kradonbok (*Careya sphaerica* roxb) leaves. Kasetsart J (Nat Sci) 2004;38:8-14.
6. Bellavite P, Andrioli G, Guzzo P, Arigliano P, Chirumbolo S, Manzato F, *et al*. A colorimetric method for the measurement of platelet adhesion in microtiter plates. Anal Biochem 1994;216:444-50.
7. Wong WT, Ismail M, Imam MU, Zhang YD. Modulation of platelet functions by crude rice (Oryza sativa) bran policosanol extract. BMC Complement Altern Med 2016;16:252.
8. Khobjai W, Suthivattanakul O, Panuwattanawong S. Phytochemical constituents and cholinesterase inhibitory activities of Millingtonia hortensis. Walailak J Sci Tech 2018;15:589-97.
9. Shealy YF, Krauth CA. Synthesis and antineoplastic evaluation of alpha-substituted alkanesulfonates: Analogues of clomesone. J Pharm Sci 1993;82:1200-4.
10. Gomha SM, Abdallah MA, Al-Showiman SS, Morad MA, Mabkhot YN. Synthesis of new pyridopyrimidinone-based thiadiazoles and pyrazolines as potential anti-breast cancer agents.

Biomed Res 2017;28:9903-9.

11. Kesternich V, Fehrmann MP, Ortíz S, Verdugo F, Brito I, Bolte M, *et al.* Synthesis, spectroscopic characterization and X-ray analysis of 6-nitroquinazoline-2,4(1H,3H)-dione. J Chil Chem Soc 2013;58:1817-9.
12. Krishnan SK, Ganguly S, Veerasamy R, Jan B. Synthesis, antiviral and cytotoxic investigation of 2-phenyl-3-substituted quinazolin-4(3H)-ones. Eur Rev Med Pharmacol Sci 2011;15:673-81.
13. Chandler JD, Min E, Huang J, McElroy CS, Dickerhof N, Mocatta T, *et al.* Antiinflammatory and antimicrobial effects of thiocyanate in a cystic fibrosis mouse model. Am J Respir Cell Mol Biol 2015;53:193-205.
14. Saundane AR, Verma VA, Katkar VT. Synthesis and antimicrobial and antioxidant activities of some new 5 (2 Methyl 1H indol 3 yl) 1, 3, 4 oxadiazol 2 amine derivatives. J Chem 2013;2013:1-9.
15. Anilkumar GN, Selyutin O, Rosenblum SB, Zeng QB, Jiang YH, Chan TY, *et al.* II. Novel HCV NS5B polymerase inhibitors: Discovery of indole C2 acyl sulfonamides. Bioorg Med Chem Lett 2012;22:713-7.
16. Dropinski JF, Akiyama T, Einstein M, Habulihaz B, Doebber T, Berger JP, *et al.* Synthesis and biological activities of novel aryl indole-2-carboxylic acid analogs as PPARgamma partial agonists. Bioorg Med Chem Lett 2005;15:5035-8.
17. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res 2010;89:219-29.
18. Tomaiuolo M, Brass LF, Stalker TJ. Regulation of platelet activation and coagulation and its role in vascular injury and arterial thrombosis. Interv Cardiol Clin 2017;6:1-12.
19. Nurden AT. Platelets, inflammation and tissue regeneration. Thromb Haemost 2011;105 Suppl 1:S13-33.
20. Falanga V. Chronic wounds: Pathophysiologic and experimental considerations. J Invest Dermatol 1993;100:721-5.
21. Hong JH, Manochai B, Trakoontivakorn G, Na Thalang V. Fibrinolytic activity of Thai indigenous vegetables. Kasetsart J (Nat Sci) 2004;38:241-6.
22. Olas B, Wachowicz B, Stochmal A, Oleszek W. Inhibition of blood platelet adhesion and secretion by different phenolics from Yucca schidigera Roezl. bark. Nutrition 2005;21:199-206.
23. Nardini M, Natella F, Scaccini C. Role of dietary polyphenols in platelet aggregation. A review of the supplementation studies. Platelets 2007;18:224-43.
24. Sukati S, Khobjai W. *In vitro* antiplatelet and anticoagulant activity of indigenous vegetables from Southern Thailand. Int J Appl Pharm 2021;13:38-42.