Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Inhibition of human platelet aggregation by Choisyaternatine isolated from *Choisya ternata* (Rutaceae)

Ikarastika Rahayu Abdul Wahab [1,*], Nikki S. H. Wong [2], Maria J. Santoz-Martinez[2,3] and Fabio Boylan [2]

[1]Faculty of Agro-based Industry, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia
[2]School of Pharmacy and Pharmaceutical Science, Trinity College, Dublin 2, Ireland
[3]School of Medicine, Trinity College Dublin, Dublin 2, Ireland

Received 21 December 2020
Accepted 25 June 2021
Online 31 December 2021

Keywords:
Choisya ternata, platelet aggregation, choisyaternatine, skimmianine

☒*Corresponding author:
Dr. Ikarastika Rahayu Abdul Wahab
Department of Agro-based Industry,
Faculty of Agro-based Industry,
Universiti Malaysia Kelantan, 17600
Jeli, Kelantan, Malaysia
Email: ikarastika@umk.edu.my

Abstract

A newly reported alkaloid was isolated from the hexane extract of *Choisya ternata* Kunth. (Rutaceae) leaves, identified as 6,8-Dimethoxy-7-(3-methylbut-2-enyloxy)quinolin-2-ol (choisyaternatine) (A), together with the known compounds skimmianine (B), lup-20(29)-en-3β-ol (lupeol) (C) and tecleamaniensine A (D). Compounds C and D are reported for the first time in this species. The structures of isolated compounds were elucidated by extensive spectroscopic analyses and comparison with previously reported spectroscopic data. The structure of choisyaternatine was further confirmed based on X-ray data analysis. Anti-platelet activity of hexane extract, choisyaternatine and skimmianine was also evaluated. The extract was able to significantly inhibit collagen-induced platelet aggregation at all concentrations tested (25, 50 and 100 µg/ml). The new compound, choisyaternatine, has shown a significant inhibition of collagen-induced platelet aggregation demonstrated in a concentration dependent manner from 250 µM. The concentrations which inhibit 50% of platelet aggregation (IC_{50}) were found to be 564 µM for skimmianine and 698 µM for choisyaternatine. This is also the first report for the antiplatelet activity for this plant and these alkaloids using human platelets.

© 2021 UMK Publisher. All rights reserved.

1. INTRODUCTION

Cardiovascular diseases (CVD) including thrombosis, stroke, ischemic, and coronary heart diseases, are a leading cause of mortality, accounting for around 30% of global deaths (Kim *et al.*, 2014; Alañón *et al.*, 2019). Among all above, thrombosis is closely related to activated platelet adhesion, aggregation, secretion functions, and activation of intrinsic and extrinsic coagulation systems, which cause blood coagulation and fibrin formation (Kim *et al.*, 2014). Most acute coronary syndromes are caused by platelet aggregation and subsequent thrombus formation in areas of ruptured atheromatous plaques (Kim *et al.*, 2014). Therefore, inhibiting platelet function represents a promising approach for preventing thrombosis (Kim *et al.*, 2014). Besides, it has also been determined that platelets represent the bridge between inflammation and thrombosis, which are fundamental processes in the development of atherothrombosis (Alañón *et al.*, 2019). Not only functioning in haemostasis and thrombosis, the multifunctional role of platelets in pathophysiology is also well established (Surin *et al*., 2008).

Platelets are also involved in maintaining of vascular tone, clot retraction, vessel constriction and repair, immune process mainly in fighting microbial infection, host defence and tumour angiogenesis and metastasis (Harrison, 2005; Katoh, 2009; Broos *et al.*, 2011). The search for potent antiplatelet drugs is continuously carried out in most laboratories, basically to substitute the known acetylsalicylic acid (aspirin). Aspirin and clopidogrel for oral administration and glycoprotein IIa/IIIb antagonists (abciximab, eptifibatide, tirofiban, etc.) for injection are commonly used antiplatelet drugs, but they have several clinical disadvantages including gastrointestinal side-effects, hemorrhage and thrombocytopenia (Kim *et al.*, 2014; Moharam *et al.*, 2010).

With this regards, much attention has been given to the development of dietary supplements or herbal medicines for prevention or treatment of cardiovascular diseases for their merits in safety (Kim *et al.*, 2014). This can be done by targeting platelet aggregation using different inducers. A modulation of platelet activity, particularly by inhibition of platelet-collagen interaction can lead to the discovery of natural products with this activity (Amrani *et al.*, 2009; Surin *et al.*, 2008). All around

eISSN Number: 2462-2389 © 2021 UMK Publisher. All rights reserved.

the world, continuous research is being conducted into phytochemicals with antiplatelet properties. These are considered to be therapeutically better, have more effective anti-platelet agents, multiple targets, and without so many side-effects (Bijak *et al.*, 2019). Based on the previous report for skimmianine, it is worth to test the compounds isolated from this species, especially alkaloids, for their antiplatelet activity using human blood. In the present study, skimmianine is one of the alkaloids that has been isolated from *Choisya ternata* leaves extracts.

Choisya ternata Kunth. (Rutaceae) is a bushy shrub with the maximum height of 2 meters. It is widely cultivated as an ornamental shrub with persistent leaves and white flowers resembling those of orange trees. It's commonly known as "Hierba del Clavo" and 'Mexican orange' because it is originated and cultivated in the central and southern mountains of Mexico (Dreyer *et al.*, 1972; Creche *et al.* 1993).

The first pharmacological use for this plant was in 1895. Boudoresques was the first to discover the medicinal effect of the aqueous extracts of leaves by experimenting on himself, which later he found them tonic and appetizing. Later, in 1923, Standley reported that Mexican people employed infusion of leaves for their antispasmodic and 'stimulative properties' (Creche *et al.*, 1993). *Choisya ternata* was later included in the Pharmacopoeia of Mexico (Respaud *et al.*, 1997).

Previous phytochemical studies have shown that *C. ternata* contains numerous quinoline alkaloids identified as skimmianine, choisyine, evoxine, kokusaginie, dictamnine and lunacrine (Creche *et al.*, 1993; Sejourne *et al.*, 1981), which derived from anthranilic acid. The biosynthetic pathway of furoquinolines has been studied in different Rutaceae, including *C. ternata* (Grundon *et al.*, 1974), and the general scheme given in previous studies by Petit-paly and co-workers in 1989 and 1993 fits with *C. ternata* (Creche *et al.*, 1993).The furoquinoline alkaloid dictamine has been shown to be a precursor of the dioxygenated alkaloid, particularly skimmianine (Collins *et al.*, 1972).

Besides, two dihydrofuroquinoline alkaloids, balfourodinium and platydesminium, have been isolated from the leaves extracts (Sejourne *et al.*, 1981; Rideau *et al.*, 1979; Creche *et al.*, 1987; Montagu *et al.*, 1986). In addition to that, 7-isopentenyloxy-γ-fagarine has been isolated and eight new members of the furoquinoline family have been synthesised from this plant (Boyd *et al.*, 2007).

.

2. MATERIALS AND METHODS

2.1 Plant materials

Leaves of *C. ternata* Kunth. were collected from the Trinity College Botanical Garden, Dartry, Dublin in October 2008 (living plant accession number 19850023) and its voucher specimen (SW 10-52) was deposited in the Herbarium of Trinity College, Dublin. The sample was identified by Dr. Steve Waldren from the Botany Department, Trinity College, Dublin, Ireland.

2.2 General

Through the TLC, the presence of terpenes, phenolics and flavonoids was indicated by the formation of coloured spots after heating on a hot plate for 5-10 minutes and the Dragendorff reagent is used to detect the presence of alkaloids.

The structural elucidation of the isolated compounds was performed by means of the analysis of their spectrums, mass spectra, H1 Nuclear Magnetic Resonance (400 and 600 MHz), Carbon-13 Nuclear Magnetic Resonance (100 and 150 MHz). The NMR spectra were recorded on a BRUKER TOPSPIN 2.1 NMR System with deuterated chloroform, deuterated methanol or deuterated dimethyl sulfoxide as solvents. Chemical shifts are reported as δ (ppm) values, and the coupling constants are given in Hz. Both uni- and bi-dimensional techniques NMR spectra (1H-1H COSY, HMBC, HMQC) were also measured by BRUKER TOPSPIN 2.1 NMR System using standard Bruker pulse sequences. Melting points were determined with SMP-1 Stuart Scientific, UK. Optical rotations were measured in a 1 decimeter tube using an Alltech AA-55 polarimeter from Optical Activity Ltd. UV analysis was performed with Cary 300 Scan UV-Visible spectrophotometer and Varian-CaryWinUV program.

The FT-IR spectroscopy was recorded in KBr discs on a Perkin-Elmer Spectrum 100 FT-IR Spectrometer (Perkin-Elmer Spectrum). Electrospray ionization (ESI) mass spectroscopy data was collected using a TOFMS (Time of Flight-Mass Spectrometer, LCT Premier) instrument supplied by Waters Corp. using Leucine Enkephalin (Leu-Enk) as internal lock mass reference. All data, including simulated isotope patterns and molecular weight calculation were processed using Masslynx v4.0 (Waters Corp.) data analysis software. An infusion of standard sodium formate (HCOONa), 10% formic acid / 0.1M NaOH/acetonitrile, 1:1:8, v/v/v) solution was used, producing a mass calibration between 100-3000. Samples were introduced into the MS via a Waters Alliance 2690 HPLC with autosample mode and a solvent flow rate of 200 µl/ml, while a Leu Enk solution (10 µg/ml in acetonitrile/0.1% TFA in water, 50:50, v/v) was co-injected via a micropump at 2 µl/min.

The ESI settings were set with the nebuliser gas and desolvation gas at 60 and 500 L/h, respectively. Source and desolvation gas temperature were set at 100 °C and 300 °C respectively. The ion polarity for all MS scans were recorded in positive or negative mode with voltage of the capillary tip set between 2.5-3 kV, sample cone at 30 v, extraction cone at 3 V, scan time at 0.9 sec and interscan

eISSN Number: 2462-2389 © 2021
UMK Publisher. All rights reserved.

delay set at 0.1 sec. EI mass spectra were acquired using a GCT Premier Micromass time of flight mass spectrometer (ToF). The instrument was operated in positive mode. Heptacosa (+) was used as an internal lock mass. Masses were recorded over a range of 100-600 m/z. Operating conditions were as follows; detector voltage 3000 V, source temperature 200 °C, filament energy 70 eV, trap current 99 uA. MassLynx 4.0 software was used to carry out the analysis.

Crystal data was collected by a Rigaku Saturn 724 CCD Diffractometer. A suitable crystal was selected and mounted using inert oil on a 0.3mm diameter glass fiber tip and placed on the goniometer head in a 123K N2 gas stream. The data was collected using Crystalclear-SM 1.4.0 software. Data integration, reduction and correction for absorption and polarization effects were all performed using Crystalclear-SM 1.4.0 software. Space group determination, structure solution and refinement were obtained using Bruker Shelxtl* Ver. 6.14 software.

The structure was solved with Direct Methods using the SHELXTL program and refined against IF2 I with the program XL from SHELX-97 using all data. Non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms were placed into geometrically calculated positions and refined using a riding model. The Software Reference Manual, version 5.625; Bruker Analytical X-Ray Systems Inc.: Madison, WI, 2001 and Sheldrick, G. M. SHELXTL were used for data collection, processing, structure solution and refinement. The crystal data from X-ray analysis was deposited in the Cambridge Crystallographic Data Centre (CCDC).

2.3 Extraction and isolation

The extraction of leaves of *C. ternata* was performed using Soxhlet apparatus. The solvents used for the extraction, chromatographic procedures and reactions were analysis grade always, exception to ethanol, which was the commercial grade 96º GL. Activated charcoal (Sigma) was also used to remove chlorophyll, when needed.

800 g ground dried leaves of *C. ternata* were extracted using Soxhlet apparatus with ethanol for 72 hours. The crude ethanol extract of *C. ternata* leaves was then treated overnight with activated charcoal (Sigma) to eliminate chlorophyll.

The crude extract (102.01 g) was subjected to liquid-liquid partition yielding hexane (42.88 g), dichloromethane (9.73 g), ethyl acetate (7.02 g) and butanol (13.91 g) extracts. The hexane extract was introduced to a column chromatography (CC) over silica gel (70-230 mesh, Merck) eluted with hexane:ethyl acetate:methanol gradient system to give 6 junctions. Junction 4 and 5 (12.31g) were combined and subjected to another CC eluted with the same mobile phase to give 13 junctions. Junction 5 and 7 and 11 were continuously cleaned with methanol to give a clear crystalline solid choisyaternatine (A, 252 mg) and yellowish precipitate skimmianine (B, 126 mg), respectively.

Junction 2 was purified by CC eluted with the same mobile phase as above to give 8 junctions. Junction 2 was cleaned continuously with methanol to give white precipitate lupeol (C, 52 mg). Junction 6 from the first column chromatography was introduced to another CC over silica gel eluted with hexane:ethyl acetate:methanol gradient system to give 15 junctions. Junction 6 was observed to contain a yellowish gum tecleamaniensine A (D, 18 mg). All the isolated and identified compounds from the hexane extract are summarised in Figure 1 (A-D).

2.4 Crystallography data of A

Crystallographic data of compound A has been deposited in the Cambridge Crystallographic Data Center as supplementary publication number CCDC 865486. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1 EZ, UK, (Fax: +44/1223/336033 or email: deposit@ccdc.cam.ac.uk).

2.5 Antiplatelet assay

All subjects gave their informed consent for inclusion before they participated in the study. The study was performed in accordance with the Guidelines for Good Research Practice, and was approved by the Research Ethics Committee at the Trinity College Dublin, Ireland.

2.6 Statistical analysis

In experiments using light aggregometer, data from at least three independent experiments were analysed using GraphPad Prism 5 software. All means are reported with standard deviation. Paired Student's t-test, One-way analyses of variance (ANOVA) and Dunnett's multiple comparisons post-test were performed where appropriate. Statistical significance was considered when $p<0.05$. The IC_{50} (concentration which inhibit 50% of platelet aggregation) was calculated from the dose-response curves using GraphPad Prism 5 software.

3. RESULT AND DISCUSSION

3.1 Phytochemical study

Five identified compounds were isolated from the hexane extract of *Choisya ternata* leaves identified as 6,8-Dimethoxy-7-(3-methylbut-2-enyloxy) quinolin-2-ol (choisyaternatine) (A), skimmianine (B), lup-20(29)-en-3β-ol (lupeol) (C) and tecleamaniensine A (D). Compound A was isolated for the first time in the plant species, while compounds C and D are the known compound and reported for the first time in this species.

eISSN Number: 2462-2389 © 2021
UMK Publisher. All rights reserved.

Choisyaternatine (A), colourless crystalline solid; R_f: 0.44 (hexane:ethyl acetate; 6:4); mp: 88-92˚C; 0.0 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 241, 300, 340; IR ν_{max}: 3083, 2942, 1715, 1563, 1406, 1272 cm^{-1} ; EI-MS for $C_{16}H_{19}NO_4$ m/z; 290 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 1.72 (3H, s, H-4'), 1.78 (3H, s, H-5'), 3.90 (3H, s, OCH_3-9), 4.04 (3H, s, OCH_3-10), 4.65 (2H, d, J=7.3 Hz, H-1'), 5.57 (1H, t, J=7.3 Hz, H-2'), 6.35 (1H, d, J=9.4 Hz, H-3), 6.68 (1H, s, H5), 7.63 (1H, d, J=9.4 Hz, H-4); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 17.9 (C-4'), 25.8 (C-5'), 56.3 (C-9), 61.7 (C-10), 70.3 (C-1'), 103.6 (C-5), 114.4 (C-4a), 115.1 (C-3), 119.9 (C-2'), 139.3 (C-3'), 141.7 (C-8), 142.9 (C-4b), 143.5 (C-4), 144.9 (C-7), 150.7 (C-6), 160.6 (C2).

Skimmianine (B), yellowish precipitate; R_f: 0.51 (chloroform:methanol:water; 9:1:1); mp: 170-175˚C; 0.0 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 258, 321, 334; IR ν_{max}: 2975, 2845, 1618, 1578 cm^{-1} ; ESI-MS for $C_{14}H_{13}NO_4$ m/z; 260 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 4.02 (3H, s, OCH_3-12), 4.10 (3H, s, OCH_3-13), 4.40 (3H, s, OCH_3-11), 7.01 (1H, d, J=2.6 Hz, H-10), 7.21 (1H, d, J=9.4 Hz, H-6), 7.56 (1H, d, J=2.6 Hz, H-9), 7.98 (1H, d, J=9.4 Hz, H-5); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 56.3 (C-12), 58.5 (C-11), 61.2 (C-13), 101.5 (C-4a), 104.2 (C-10), 111.4 (C-6), 114.3 (C-3), 117.8 (C-5), 140.9 (C-8), 141.3 (C-7), 142.9 (C-9), 51.7 (C-4b), 156.7 (C-4), 163.9 (C-2). This data is in fair agreement to that reported by Cordoso-Lopes *et al.* (2010) and Brown *et al.* (1980).

Lup-20(29)-en-3β-ol (lupeol) (C), white precipitate; R_f: 0.57 (hexane:ethyl acetate; 6:4); mp: 250-252˚C; +9.09 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 244; IR ν_{max}: 3320, 2916, 2848, 1462, 1266, 718 cm^{-1} ; ESI-MS for $C_{30}H_50O_2$ protonated molecular ion m/z; 443 $[M+H]^+$; 1H-NMR ($CDCl_3$, 400 MHz) δ_H: 0.72 (1H, m, H-5), 0.79 (3H, s, H-25, CH_3), 0.82 (3H, s, H-28), 0.97 (3H, br, s, H-27), 0.99 (3H, s, H-23), 1.06 (3H, s, H-26), 1.28 (1H, m, H-9), 1.36 (2H, m, H-16), 1.39 (2H, m, H-7), 1.42 (2H, m, H-11), 1.63 (2H, m, H-2), 1.71 (3H, br, s, H-30), 1.94 (2H, m, H-21), 2.41 (1H, m, H-19), 4.10 (3H, s, H-24), 4.59 (2H, m, H-29b), 4.72 (2H, d, J=2.4 Hz, H29a); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 14.6 (C-27), 15.9 (C-25), 16.1 (C-26), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 20.9 (C-11), 25.1 (C-12), 27.4 (C-15), 27.5 (C-2), 27.9 (C-23), 29.7 (C-21), 34.3 (C-7), 35.6 (C-16), 37.2 (C-10), 38.1 (C-13), 38.7 (C-1), 38.9 (C-4), 40.0 (C-22), 40.8 (C-8), 42.8 (C-14), 43.0 (C-17), 48.0 (C-19), 48.3 (C-18), 50.5 (C-9), 55.3 (C5), 63.1 (C-24), 79.0 (C-3), 109.3 (C-29), 150.9 (C-20). This data are in agreement to that reported by Tanaka *et al.* (1988) and, Mahato and Kundu (1994).

Tecleamaniensine A (D), yellowish gum; R_f: 0.75 (chloroform:methanol:water; 9:1:1); mp: 120-125˚C; 0.0 (CH_2Cl_2); UV (CH_2Cl_2) λ_{max}: 252, 320, 333; IR ν_{max}: 2930, 2856, 1607, 1576, 1356, 1255, 1063 cm^{-1} ; ESI-MS for $C_{19}H_{21}NO_4$ m/z; 328 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H: 1.69 (3H, d, J=1.3 Hz, H-4'), 1.86 (3H, d, J=0.8 Hz, H-5'), 3.85 (3H, s, OCH_3-12), 4.00 (3H, s, OCH_3-13), 4.06 (2H, d, J=6.2 Hz, H-1'), 4.35 (3H, s, OCH_3-11), 5.25 (1H, t, J=6.3 Hz, H-2'), 7.04 (1H, d, J=2.7 Hz, H-10), 7.29 (1H, s, H-8), 7.55 (1H, d, J=2.7 Hz, H-9); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ_C: 18.2 (C-4'), 25.8 (C-5'), 27.5 (C-1'), 55. 7 (C-13), 59.4 (C-11), 61.0 (C-12), 103.5 (C-3), 105.3 (C-10), 106.2 (C-8), 113.0 (C-4a), 124.9 (C-2'), 130.4 (C-5), 130.9 (C-3'), 142.2 (C-9), 145.8 (C-4b), 145.9 (C-6), 154.9 (C-7), 159.1 (C4), 163.2 (C-2). This data is an agreement to that reported by Magadula *et al.* (2008).

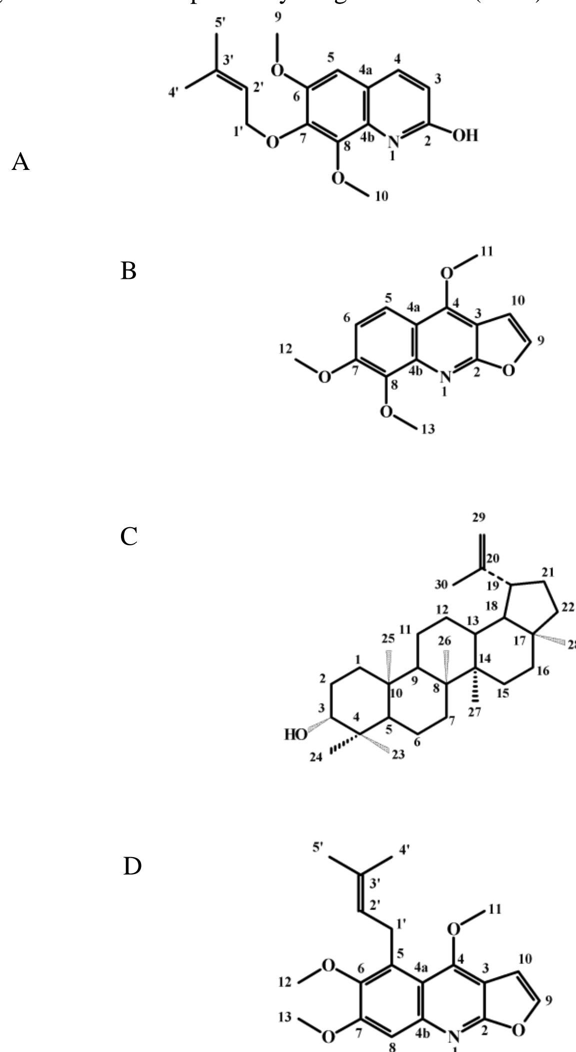

Figure 1: Compounds isolated from the hexane extract of *Choisya ternata* leaves; choisyaternatine (A), skimmianine (B), lupeol (C) and tecleamaniensine A (D).

3.2 Human platelet activity

The crude hexane, from where the alkaloids came from and compound A, B, C and D were assayed for the antiplatelet activity. The percentage of inhibition of collagen-induced platelet aggregation at various concentrations for the hexane extract and isolated compounds choisyaternatine (A), skimmianine (B), lupeol

eISSN Number: 2462-2389 © 2021 UMK Publisher. All rights reserved.

(C) and tecleamaniensine A (D) are shown in Figure 2 and 3, respectively.

First we tested the ability of the hexane extract to inhibit platelet aggregation. As shown in Figure 2, the extract was able to significantly inhibit collagen-induced platelet aggregation in a concentration-dependent manner. Following to that, the alkaloids and tritepene isolated from the hexane extract; choisyaternatine (A), skimmianine (B), lupeol (C) and tecleamaniensine A (D) were tested using the same method to evaluate their antiplatelet activity.

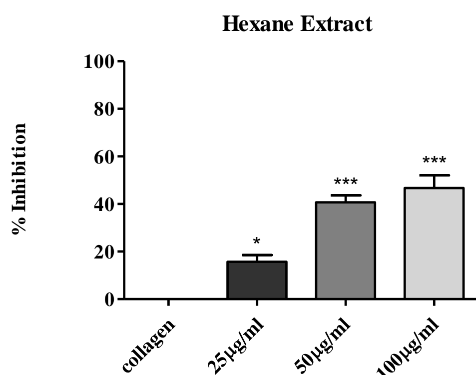

Figure 2: Quantitative analysis of the inhibitory effect on collagen-induced platelet aggregation of hexane extract with all concentrations tested. Data are expressed as mean ± S.D. *p<0.05 vs. collagen; ***p<0.001 vs. collagen.

Among all the isolated alkaloids reported in this study, only skimmianine has been previously reported as the one that inhibited platelet aggregation. Skimmianine at 100 µg/ml (equivalent to 386 µM) inhibited collagen-induced platelet aggregation (around 66%) in washed rabbit platelets (Chen *et al.*, 2000.) Here we show, for the first time, that this compound is also able to inhibit collagen-induced platelet aggregation in human platelets, together with choisyaternatine (A), lupeol (C), and tecleamaniensine A (D).

The new compound which isolated from the previous study, choisyaternatine, as well as tecleamaniensine A, showed a significant inhibition of collagen-induced platelet aggregation in a concentration dependent manner from 50-1000 µM. The concentrations which inhibit 50% of platelet aggregation (IC_{50}) were found to be 698, 564 and 719 µM for choisyaternatine, skimmianine and tecleamaniensine A, respectively. The IC_{50} for the hexane extract was much lower than these four compounds, at 50-100 µg/ml, while no significant inhibition showed by triterpene lupeol (Figure 3 A-D).

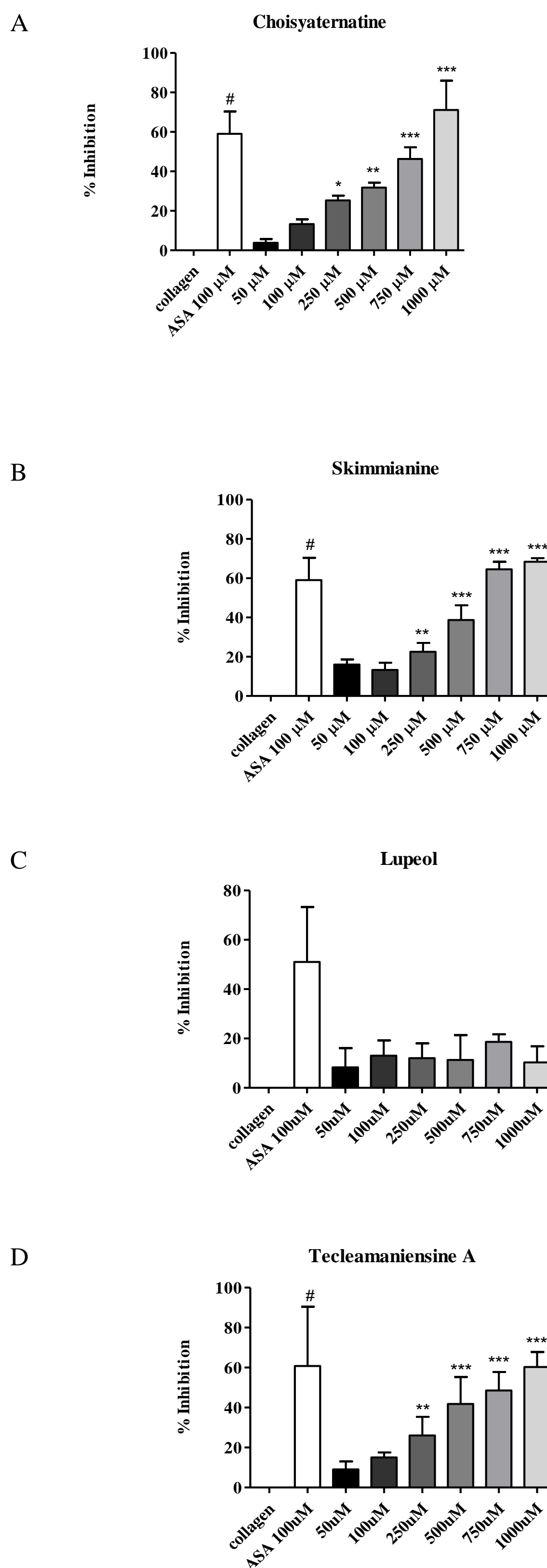

Figure 3: Quantitative analysis of the inhibitory effect on collagen-induced platelet aggregation of (A) choisyaternatine, (B) skimmianine, (C) lupeol and (D) tecleamananiensine A. Data are expressed as mean ± S.D. *p<0.05 vs. collagen; **p<0.01; ***p<0.001 vs. collagen. Acetylsalicylic acid (ASA) is included as internal control ($^{\#}$p<0.05 vs. collagen).

eISSN Number: 2462-2389 © 2021 UMK Publisher. All rights reserved.

Knowing that the synergism of the compounds in the extract could contribute to the high activity, a mixture of choisyaternatine (700 µM), skimmianine (560 µM), lupeol (>1000 µM) and tecleamaniensine A (719 µM) was tested using the same procedure. The results were able to show that the mixture of these four compounds had a higher percentage of platelet inhibition when compared to choisyaternatine (A), skimmianine (B), lupeol (C) and tecleamaniensine A (D) (Figure 4). Furthermore, the effect showed by the mixture is higher than the hexane extract, with the percentage of inhibition for the mixture was 99% compared to only 50% for the extract. This could be possibility due to the interaction with other compounds present in the hexane extract, together with the four tested compounds.

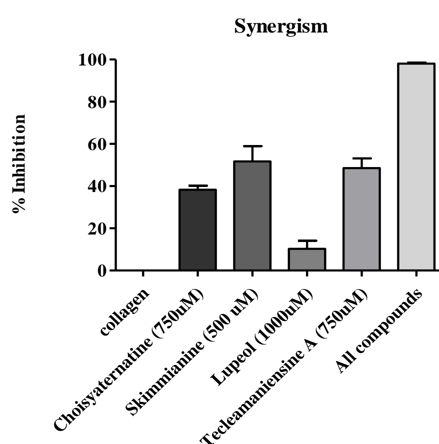

Figure 4: Quantitative analysis of the inhibitory effects on collagen-induced platelet aggregation of a mixture of choisyaternatine (A), skimmianine (B), lupeol (C) and tecleamaniensine A (D). Data are expressed as mean ± S.D. *p<0.05 vs. collagen; **p<0.01; ***p<0.001 vs. collagen. Acetylsalicylic acid (ASA) is included as internal control ($^{\#}$p<0.05 vs. collagen). Control consists in 2 µL DMSO + 2 µg/ml collagen.

The phytochemical studies yielded quinoline alkaloids and pentacyclic tritepenes (lupeol and lupeol and lup-20(29)-en-3β,24-diol) isolated from this extract. It is well known that quinoline ring system is an important structural unit widely existing in alkaloids, therapeutics and synthetic analogues with interesting biological activities. A large variety of quinoline derivatives have been used as antimalarial, anti-inflammatory, antiasthmatic, antibacterial, antihypertensive and platelet derived growth factor receptor tyrosine kinase inhibiting agents (Naik *et al.*, 2009).

Skimmianine, one of the furoquinoline alkaloids, isolated from the leaves of *Melicope confuse* (Rutaceae) was reported as showing significant antiplatelet aggregation activity (Chen *et al.*, 2000). The report was in agreement with Wu *et al.* (2003) who also reported a significant inhibitory activity of platelet aggregation induced by collagen (10 µg/ml) by skimmianine and dictamnine. At the highest concentration of 100 µg/ml, the furoquinoline alkaloids isolated from *Ruta graveolens* (Rutaceae) inhibited the platelet aggregation at 45.9±17.4 and 84.4±6.2, respectively. The most potent antiplatelet activity was shown by the quinoline alkaloid graveolinine with 72.6% of inhibition of collagen-induced platelet aggregation (Wu *et al.*, 2003).

Lupeol, isolated from *Garcinia hombroniana*, showed a moderate inhibition of whole human blood platelet-induced by collagen (2 µg/ml) with 59.0±3.3% at 100 µg/ml (Saputri and Jantan, 2012), while the one that isolated from the root of *Petasites formosanus* showed a weak inhibition against washed rabbit platelets induced by collagen (10 µg/ml) with only 3.1±2.1% at 20 µg/ml (Wu *et al.*, 2003). In this study, at the highest concentration of 1000 µM, percentage of inhibition of lupeol was only 10% (collagen 2 µg/ml) which could be due to its solubility in DMSO.

The discrepancy found between the previous and the present study may be due to the different samples and experimental conditions (concentration of collagen, incubation time etc.) For example, several studies used 10 µg/ml of collagen and other different concentrations (data not shown in the paper) (Chen *et al.*, 2000; Ching *et al.*, 2010), than the one used in this study which was 2 µg/ml.

Although additional studies are needed to investigate the contribution of the main platelet mediators involved in this process and therefore to elucidate the mechanism of action of these compounds; the effect of the well-known antiplatelet agent acetylsalicylic acid (ASA, 100 µM) was also evaluated as an internal control (Figure 3 A-D).

4. CONCLUSION

An isoprenyl quinoline alkaloid identified as choisyaternatine (A) was isolated from the hexane extract of *Choisya ternata* Kunth. (Rutaceae) leaves, together with other known compounds; skimmianine (B), lup-20(29)-en-3β-ol (lupeol) (C), lup-20(29)-en-3β,24-diol (choisyaternatine (A) was further confirmed based on X-ray data analysis.

The hexane extract, choisyaternatine (A), skimmianine (B), lupeol (C) and tecleamaniensine A (D) were evaluated its antiplatelet activity using whole human blood. In this study, the extract significantly inhibited collagen-induced platelet aggregation at all concentrations tested. Compounds C and D are reported for the first time in this species. Structures of the isolated compounds were elucidated by extensive spectroscopic analyses and comparison with previously reported spectroscopic data. Choisyaternatine and skimminanine only showed a moderate collagen-induced platelet aggregation activity in human platelets with the IC_{50} value of 564 and 698 µM,

eISSN Number: 2462-2389 © 2021 UMK Publisher. All rights reserved.

respectively, while lupeol showed no activity at all concentrations tested. However, a mixture of the alkaloids at their IC_{50} concentrations with lupeol > 1000 μM exhibited an important synergism activity, with this mixture being able to inhibit aggregation at 99% using the same concentration of collagen to induce aggregation as previous.

Additional studies are needed to investigate the contribution of the main platelet mediators involved in this process and therefore to elucidate the mechanism of action of these compounds and other extracts including their isolates.Taken together, these results highlighted the use of *Choisya ternata* extracts as a promising natural co-product with moderate antiplatelet effects, which can be used as a pharmaceutical drug or as a functional food ingredient with therapeutic applications against platelet aggregation.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Higher Education, Malaysia, Universiti Malaysia Kelantan, Malaysia and Trinity College, Dublin, Ireland. The antiplatelet assay was kindly supervised by Dr. Maria Jose Santos-Martinez and performed in the lab she shares with Dr. Marek Radomski at the School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin. The authors also would like to thank Dr. John F. Gilmer, all technicians in the School of Pharmacy and Pharmaceutical Sciences, Chemistry Department and Botany Department of Trinity College Dublin.

REFERENCES

Alañón,M. E., Palomo, I., Rodríguez, L., Fuentes, E., Arráez-Román, D. & Segura-Carretero, A. 2019. Antiplatelet Activity of Natural Bioactive Extracts from Mango (*Mangifera Indica* L.) and its By-Products. Antioxidants, 8, 517.

Amrani, S., Harnafi, H., Gadi, D., Mekhfi, H., Legssyer, A., Aziz, M., Martin- Nizard, F. & Bosca, L. 2009. Vasorelaxant and anti-platelet aggregation effects of aqueous *Ocimum basilicumextract*. Journal of Ethnopharmacology, 125, 157-162.

Bijak M, Sut A, Kosiorek A, Saluk-Bijak J. & Golanski J. 2019. Dual Anticoagulant/Antiplatelet Activity of Polyphenolic Grape Seeds Extract. Nutrient*s*, 5, 93.

Boyd, D. R., Sharma, N. D., Loke, P. L., Malone, J. F., Mcroberts, W. C. & Hamilton, J. T. G. 2007. Synthesis, structure and stereochemistry of quinoline alkaloids from *Choisya ternata*. Org. Biomol. Chem*.*, 5, 2983-2991.

Broos, K., Feys, H. B., De Meyer, S. F., Vanhoorelbeke, K. & Deckmyn, H. 2011. Platelets at work in primary hemostasis. Blood Reviews, 25, 155-167.

Brown, N., Grundon, M., Harrison, D. & Surgenor, S. 1980. Quinoline alkaloids—XXI: The 13C NMR spectra of hemiterpenoid quinoline alkaloids and related prenylquinolines. Tetrahedron, 36, 3579-3584.

Cardoso-Lopes, E. M., Maier, J. A., Silva, M. R., RegasinI, L. O., Simote, S. Y., Lopes, N. P., Pirani, J. R., Bolzani, V. S. & Young, M. C. M. 2010. Alkaloids from stems of *Esenbeckia leiocarpa* Engl. (Rutaceae) as potential treatment for Alzheimer disease. Molecules, 15, 9205-9213.

Chen, K. S., Chang, Y. L., Teng, C. M., Chen, C. F. & Wu, Y. C. 2000. Furoquinolines with antiplatelet aggregation activity from leaves of Melicope confusa. Planta Med, 66**,** 80-81.

Ching, J., Chua, T. K., Chin, L. C., Lau, A. J., Pang, Y. K., Jaya, J., Tan, C. H. & Koh, H. L. 2010. β-amyrin from *Ardisia elliptica* Thunb. is more potent than aspirin in inhibiting collagen-induced platelet aggregation. Indian J. Exp. Biol, 48**,** 275-279.

Collins, J., Donnelly, W., Grundon, M., Harrison, D. & Spyropoulos, C. 1972. Aromatic hydroxylation in quinoline alkaloids. The biosynthesis of skimmianine from dictamnine, and a convenient synthesis of furanoquinoline alkaloids. J. Chem. Soc., Chem. Commun.**,** 1029-1030.

Creche, J., Guiller, J., Andreu, F., Gras, M., Chenieux, J. & Rideau, M. 1987. Variability in tissue cultures of *Choisya ternata* originating from a single tree. Phytochemistry*,* 26**,** 1947-1953.

Creche, J., Trémouillaux-Guiller, J., Gröger, D., Chénieux, J. & Rideau, M. 1993. *Choisya ternata* (Mexican Orange): *In vitro* culture and the production of alkaloids. *In:* Bajaj, Y. P. S. (Ed.) Biotechnology in Agriculture and Forestry 24 (Medicinal and Aromatic Plants V) Heidelberg: Springer.

Dreyer, D. L., Pickering, M. V. & Cohan, P. 1972. Distribution of limonoids in the Rutaceae. Phytochemistry, 11**,** 705-713.

Grundon, M. F., Harrison, D. M. & Spyropoulos, C. G. 1974. Biosynthesis of the furoquinoline alkaloids, skimmianine, evoxine, and choisyine: mechanism of formation of the furan ring. The timing of aromatic hydroxylation and of methylation. J. Chem. Soc., Chem. Commun.**,** 51-52.

Harrison, P. 2005. Platelet function analysis. *Blood reviews*, 19, 111-123.

Jones, M., Inkielewicz, I., Medina, C., Santos-Martinez, M. J., Radomski, A., Radomski, M. W., Lally, M. N., Moriarty, L. M., Gaynor, J. & Carolan, C. G. 2009. Isosorbide-based aspirin prodrugs: integration of nitric oxide releasing groups. Journal of medicinal chemistry, 52**,** 6588-6598.

Jurasz, P., Sawicki, G., Duszyk, M., Sawicka, J., Miranda, C., Mayers, I. & Radomski, M. W. 2001. Matrix metalloproteinase 2 in tumor cell-induced platelet aggregation: regulation by nitric oxide. Cancer research, 61**,** 376.

Katoh, N. 2009. Platelets as versatile regulators of cutaneous inflammation. Journal of Dermatological Science, 53, 89-95.

Kim, D. S., Ji, H. D., Rhee, M. H., Sung, Y. Y., Yang, W. K., Kim, S. H., Kim, H. K. 2014. Antiplatelet activity of *Morus alba* leaves extract, mediated via inhibiting granule secretion and blocking the phosphorylation of extracellular-signal-regulated kinase and akt. Evidence-Based Complementary and Alternative Medicine, 639548, 11 pages.

Magadula, J. J., Kapingu, M. C., Mbwambo, Z. H. & Mulholland, D. A. 2008. Secondary metabolites from *Teclea amanuensis* (Rutaceae) from Tanzania. Natural Product Communications, 3, 1683-1686.

Mahato, S. B. & Kundu, A. P. 1994. 13C NMR Spectra of pentacyclic triterpenoids--a compilation and some salient features. Phytochemistry, 37, 1517-1575.

Medina, C., Jurasz, P., Santos-Martinez, M. J., Jeong, S. S., Mitsky, T., Chen, R. & Radomski, M. W. 2006. Platelet aggregation-induced by caco-2 cells: regulation by matrix metalloproteinase-2 and adenosine diphosphate. Journal of Pharmacology and Experimental Therapeutics*,* 317**,** 739-745.

Moharam, B. A., Jantan, I., Jalil, J. & Shaari, K. 2010. Inhibitory effects of phylligenin and quebrachitol isolated from *Mitrephora vulpinaon* platelet activating factor receptor binding and platelet aggregation. Molecules, 15, 7840-7848.

Montagu, M., Levillain, P., Chenieux, J. C. & Rideau, M. 1986. Dosage d'alcaloides dihydrofuro[2,3-b]quinoleinium dns des tissus végétaux *in vitro* par chromatographie sur couche mince de gel de silice et fluorodensitométrie. Journal of Chromatography *A,* 351**,** 144-149.

Naik, H. R. P., Naik, H. S. B., Naik, T. R. R., Naika, H. R., Gouthamchandra, K., Mahmood, R. & Ahamed, B. M. K. 2009. Synthesis of novel benzo[h]quinolines: Wound healing, antibacterial, DNA binding and *in vitro* antioxidant activity. European Journal of Medicinal Chemistry*,* 44**,** 981-989.

Radomski, A., Jurasz, P., Alonso-Escolano, D., Drews, M., Morandi, M., Malinski, T. & Radomski, M. W. 2005. Nanoparticle-induced platelet

eISSN Number: 2462-2389 © 2021 UMK Publisher. All rights reserved.

aggregation and vascular thrombosis. British journal of pharmacology, 146, 882-893.

Radomski, M. & Moncada, S. 1983. An improved method for washing of human platelets with prostacyclin. Thrombosis research, 30, 383.

Respaud, M. J., Moulis, C., Fouraste, I. & Bessière, J. M. 1997. Essential Oil Composition of *Choisya ternata* Kunth (Rutaceae) Leaves. Journal Of Essential Oil Research, 9, 475-476.

Rideau M, V. C., Hibon P, Chénieux J-C, Maupas P. & Viel C. 1979. Alcaloïdes dihydrofuroquinoleiques de quelques Rutaceae: isolement, structure, proprietes biologiques. Phytochemistry, 18, 155-159.

Saputri, F. C. & Jantan, I. 2012. Inhibitory activities of compounds from the twigs of *Garcinia hombroniana* Pierre on Human Low-density Lipoprotein (LDL) Oxidation and Platelet Aggregation. Phytotherapy Research.

Sejourne, M., Viel, C., Bruneton, J., Rideau, M. & Chenieux, J. 1981. Growth and furoquinoline alkaloid production in cultured cells of *Choisya ternata*. Phytochemistry, 20, 353-355.

Surin, W. R., Barthwal, M. K. & Dikshit, M. 2008. Platelet collagen receptors, signaling and antagonism: emerging approaches for the prevention of intravascular thrombosis. Thrombosis Research, 122, 786-803.

Tanaka, R., Tabuse, M. & Matsunaga, S. 1988. Triterpenes from the stem bark of *Phyllanthus flexuosus*. Phytochemistry, 27, 3563-3567.

Wu, T. S., Shi, L. S., Wang, J. J., Iou, S. C., Chang, H. C., Chen, Y. P., Kuo, Y. H., Chang, Y. L. & Teng, C. M. 2003. Cytotoxic and antiplatelet aggregation principles of *Ruta graveolens*. Journal Chinese Chemical Society Taipei, 50, 171-178.

eISSN Number: 2462-2389 © 2021 UMK Publisher. All rights reserved.