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Evaluation of the ethanolic extract of some medicinal plants on platelet function in COVID-19 patients

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Abstract

Coronavirus disease-2019 (COVID-19) caused by SARS-CoV-2 is an ongoing viral pandemic marked by dysfunctional platelet responses resulting in increased risk of thrombotic events. Indeed, platelet hyper-reactivity has been shown to be common in COVID-19 patients. The antiviral effect of a few natural products has been documented by several studies, but none have focus on their effect on platelet function in COVID-19 patients. The aim of this work was to evaluate *in vitro* the effect of some medicinal plants on platelet activation and aggregation in COVID-19 patients and the possible mechanism. Platelet hyper-reactivity was assessed by measurements of aggregation and translocation/phosphorylation of PKCδ on Tyr^{311} following stimulation by collagen**,** while *C. Longa* ethanolic extract failed to inhibit platelet hyper-reactivity observed in COVID-19 patients. The present study reports that ethanolic extracts of *L. Angustifolia* and *R. Officinalis* could significantly reduce platelet response to low concentrations of collagen. These findings confirmed the link between the traditional medicinal plants used and the results of the scientific research studies. In order to explore, maximum benefits of the present study, it is suggested to conduct further scientific studies using randomized controlled trials.

Keywords: Platelets; COVID-19; *C. Longa*; *L. Angustifolia*; *R. Officinalis*; PKC delta

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1. Introduction

The Coronavirus Disease 2019 (COVID-19) is an outbreak caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), still spreading and has led to unprecedented health emergency all over the world. It is characterized by an acute respiratory distress syndrome (ARDS) accompanied with clinical pathologies, including various coagulopathies that may be associated by hyper-coagulation and platelet hyper-activation [1]. Therefore, the involvement of platelet function in the pathophysiology of COVID-19 is well documented.

While vaccination campaign is underway, only few treatments showed efficacy against SARS-CoV-2 which is probably the biggest challenge for public health systems in most countries [2].

The effective antiviral activities of natural products have been proved in different studies [3, 4]. *Curcuma Longa*, named "Kharkoum" in Morocco, a rooted plant in the ginger family, has a long history of medicinal use in different folklore and traditional medicine [5] and has become the first choice for alternative medicine due to its anti-inflammatory and antioxidant effects [6].

Recently, it has been shown that *Curcuma longa* enhances the secretion of IFN-γ, a key regulator of human immune system, suggesting its potential use as an immune-boosting functional food ingredient [7]. In Colombia, it is traditionally consumed as a circulatory stimulant and pro-thrombosis [8]. *Lavandula angustifolia* "Khouzama", a plant

found in many countries, is known for its anti-fungal, antimicrobial, and anti-protozoan qualities [9, 10]. *Rosmarinus Officinalis* L. "Azir" is a widely known species for its medicinal uses. *Rosmarinus Officinalis* L., that are largely distributed in habitats from Southern and Northern Africa, Western Asia, Anatolia and the Mediterranean basin [11]. Its leaves have significant therapeutic potential against a wide range of diseases such as diabetes mellitus, stomach disorders, respiratory and inflammatory diseases [12]. In folk medicine Rosemary is used for the prevention and the treatment of diabetes and cardiovascular diseases [13].

To the best of our knowledge, no studies have evaluated the effect of medicinal plant extracts on platelet function in COVID-19 patients.

The study was performed in accordance with the Declaration of Helsinki and a written consent form was signed by all patients included or their trusted relatives at the time of enrolment (SARCODO 2020-A01048-31A, NCT04624997). All included patients, hospitalized or not, presented a confirmed diagnosis of COVID-19, using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay on nasopharyngeal swab samples as previously described.

2. Materials and methods

2.1. Plant materials

Three medicinal/food plants were selected in this study known for their higher phenolics contents, antioxidant capacity and lower or inexistent toxicity. The plant species were botanically authenticated by Prof. Hamid Khamar (Department of Botany and Plant Ecology, Scientific Institute, Mohammed V University, Rabat, Morocco). The results of spectrophotometric analysis of these plant extracts are shown in Table 1. The voucher numbers of the plants are as follows: RAB112424: *Lavandula Angustifolia*, RAB112425: *Rosmarinus Officinalis* L., and RAB112426: *Curcuma Longa*.

2.2. The preparation of the plant extracts

The samples were coarsely powdered and packed into a Soxhlet column and extracted with 70% v/v ethanol in water at 75–79°C for 15 hours. The extract obtained was evaporated at 45°C. Afterwards, the ethanol extract was successively separated by a series of increasing polar solvents (hexane, ethyl acetate, butanol and distilled water) according to the method previously published [14].

2.3. Patients

The inclusion criteria included patients, hospitalized or not, presented a confirmed diagnosis of COVID-19, using a reverse transcriptase–polymerase chain reaction (RT-PCR) assay on nasopharyngeal swab samples who were admitted to Cheikh Zaid Hospital from December 7, 2020 to March 8, 2021. The exclusion criteria included incomplete medical records.

The recruitment was approved by the Ethics Committee of Cheikh Zaid Hospital (CEFCZ/PR/2020/PR04) and complies with the Declaration of Helsinki. All participants gave their written informed consent. Table 2 describes the clinical characteristics of COVID-19 patients.

2.4. Confocal Microscopy Studies

The platelets were stimulated with collagen in the presence of ethanolic extract or its vehicle for 5 minutes at 37ºC, fixed for 30 minutes with 2% (v/v) paraformaldehyde in phosphate-buffered saline, washed twice, and allowed to immobilize on poly-L-lysine-coated coverslips overnight at 4ºC. Adhered platelets were permeabilized for 20 minutes with 0.1% Triton X-100 containing 2% bovine serum albumin. The platelets were then incubated for 3h with the rabbit anti-human polyclonal anti-PKCδ antibody (Abcam) and the mouse anti-human monoclonal antibody against α-tubulin (Santa Cruz Biotechnology), washed and labelled with anti-rabbit IgG Alexa-555 and anti-mouse IgG Alexa-488 secondary antibodies for 1h at room temperature. Coverslips were mounted on microscopic slides and series of fluorescent confocal images (Z-stacks) were acquired with a LSM 510 confocal microscope (Zeiss, Oberkochen, Germany).

2.5. PKCδ Phosphorylation

The platelets were stimulated with collagen in the presence of ethanolic extract or its vehicle for 5 minutes under shear conditions at 37ºC. The reaction was stopped by adding the appropriate volume of 4×Laemmli buffer. The platelet lysates were heated for 5 minutes at 95ºC and then stored at -20°C for further analysis by SDS-PAGE. Proteins were resolved in 8% SDS-PAGE gels and transferred onto nitrocellulose membranes, blocked with 5% non-fat dry milk for 1 hour, washed three times with TBS/Tween (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 0.1% Tween-20) and incubated overnight at 4ºC with antibodies against phospho-PKCδ Tyr311 (Cell Signaling Technology). Following washing steps, membranes were labelled with horseradish peroxidase-conjugated secondary antibody for 1 hour, washed and bound peroxidase activity was detected by enhanced chemiluminescence (PerkinElmer Life Sciences).

To assess equal amounts of protein loading, membranes were stripped and blotted for total PKCδ (Cell Signaling Technology).

2.6. Platelet aggregation assay

Aggregation was monitored on an eight-channel optical aggregometer (SD Medical Innovation, Frouard, France) at 37°C under stirred conditions as previously described [15-17]. Briefly, the samples were stimulated with collagen (Chrono-Log, USA) at concentrations of 0.5 and 5 µg/mL. The platelet aggregation was monitored following the addition of an appropriate concentration of collagen and recorded until trace stabilization and light transmission was measured at the time of maximum aggregation.

2.7. Statistical analysis

The results are presented as mean ± SD of at least 3 independent experiments. Statistical comparisons were done using a one-way ANOVA, followed by a Dunnett's test for comparison against a single group. Data with $P \leq 0.05$ were considered statistically significant.

Table 1: Phenolic content of methanolic extracts of the studied medicinal and food plants

Medicinal Plant	Total phenols (mg GAE g^{-1} DW)	Ortho-diphenols (mg GAE g^{-1} DW)	Flavonoids (mg CATE g^{-1} DW)	Tannins (mg ECE g^{-1} DW)
R. Officinalis	48.48±0.13	133.88±1.78	49.14±0.83	18.10±0.77
C. Longa	13.26±1.77	39.16±0.93	45.39±1.06	95.27±0.16
L. Angustifolia	41.92±0.64	108.84±0.67	35.72±0.70	23.58±0.44

Values are presented as mean ± SD (n = 3) for each phenolic group.
Values in bold represent the highest for each parameter assessed.

Table 2: Clinical characteristics of COVID-19 patients

Index	Healthy donors	COVID-19 patients	p value
N° of patients	10	10	
Female/Male	5/5	5/5	--
Age, years	50.32±12.24	53±16.27	0.82
Weight, kg	79.44±26.82	82.67±13.94	0.94
Platelet number x 10^9/L	238±75.59	187.5±96.61	0.21
Lymphocyte number x 10^9/L	1.19±0.87	1.08±0.53	0.36
ALT, U/L	20.54±5.43	34.22±15.48	0.01
AST, U/L	19.62±4.40	36.17±14.81	0.01
LDH, U/L	362.89±134.37	616.30±192.36	0.01
C-reactive protein, mg/L	8.39±4.11	22.64±12.43	0.05
D-dimers, mg/L	0.56±0.38	1.46±0.62	0.01

Data are presented as mean ± standard deviation. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase. Statistical analysis: unpaired Student t test was used to calculate p values. Bold numbers indicate statistical significance at ≤ 0.05

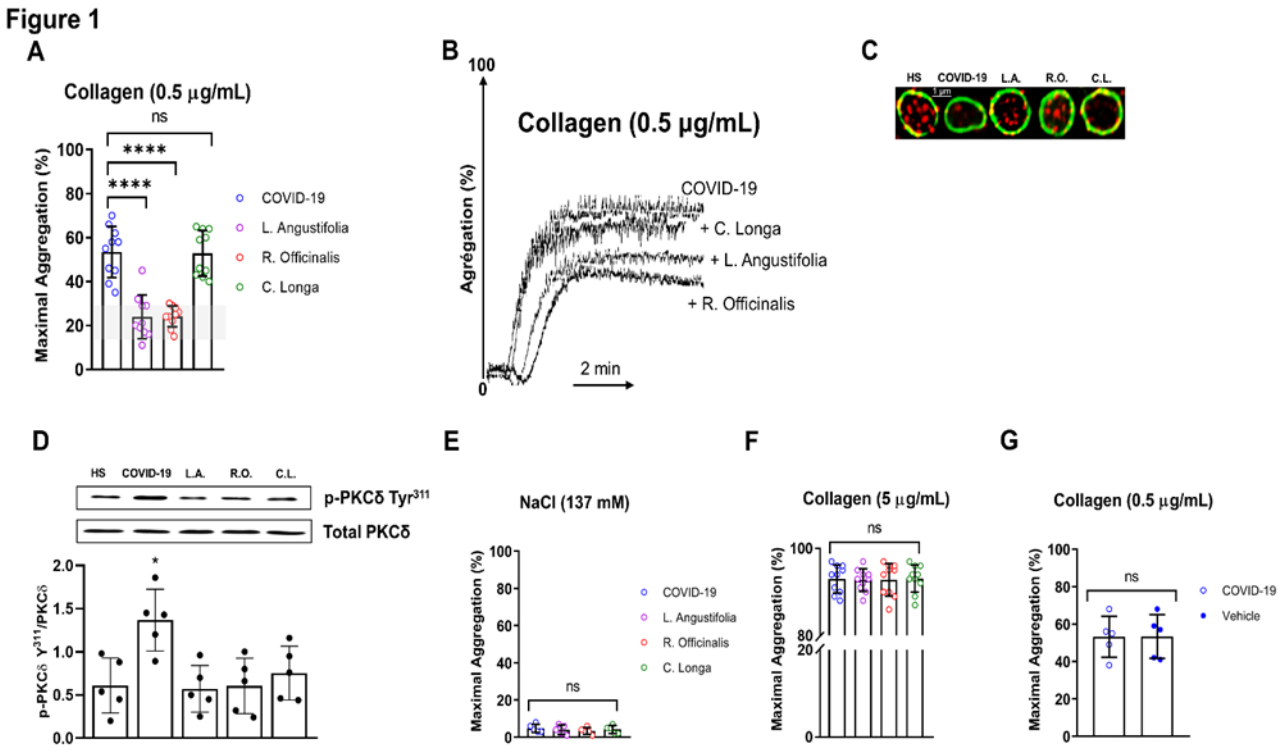

Fig. 1: *L. Angustifolia* and *R. Officinalis*-treatment reduce platelet hyper-reactivity to collagen. Platelets were isolated from Healthy Donors (n=10) and severe COVID-19 patients (n=10, blue dots). Quantification of maximal aggregation of platelets from COVID-19 patients pre-treated (or not) with ethanolic extract of *L. Angustifolia* (n=10, mauve dots), *R. Officinalis* (n=10, red dots) and *C. Longa* (n=10, green dots) for 5 min at 37°C in response to low (A) and high (F) doses of collagen. Quantification of Healthy Controls is shown as grey overlay. (B) Representative aggregation traces under continuous stirring at 37°C. (C) *L. Angustifolia* and *R. Officinalis* inhibit membrane translocation of PKCδ observed in platelets from COVID-19 patients in response to a low dose of collagen. Representative confocal microscopy images showing platelets double stained for PKCδ (red) and α-tubulin (green) taken at 63X magnification. (D) PKCδ phosphorylation on Tyr^{311} residue is increased in response to collagen in patients with severe COVID-19 and pretreatment of platelets with ethanolic extracts of *L. Angustifolia*, *R. Officinalis* and *C. Longa* for 5 min at 37°C. Platelet lysates were analyzed by SDS-PAGE for *P*-PKCδ Tyr^{311}. Total PKCδ was evaluated in each condition. Immunoblot (upper) representative of 5 donors. Densitometric (lower) analysis of *P*-PKCδ Tyr^{311} normalized to total PKCδ was performed, data were expressed as relative Optical Density (n=5). Quantification of maximal aggregation of platelets preincubated with NaCl (n=5) (E) and complete buffer/vehicle (n=5) (G). Data are represented as mean ± SD. Statistical analysis: One-way ANOVA with subsequent Dunnett's-t-test for comparison against a single group. *P<0.05 and ****P<0.0001

3. Results & Discussion

Several study groups [18-20] including ours [15, 21, 22] have reported increased platelet activation, aggregation and platelet-leukocyte aggregates in COVID-19 compared with those in healthy blood donors. Other studies reported the presence of platelets in thrombi found in multiple organs of COVID-19 autopsy cases [23, 24] while a recent study has shown the presence of SARS-CoV-2 virions in platelets [25]. These studies demonstrate that platelet reactivity is enhanced during SARS-CoV-2 infection and posit that platelet hyper-reactivity may be a primary driver of thrombosis in patients with severe COVID-19, contributing to organ failure and death. Whether platelet hyper-reactivity can be reversed following treatment with ethanolic extract of some medicinal plants was addressed in our study. The present study demonstrated the in vitro efficacy of *C. longa*, *L. angustifolia* and *R. officinalis* in the inhibition of platelet hyper-reactivity during SARS-CoV-2 infection.

These findings confirm that platelets from patients with COVID-19 are hyper-activated (Figure 1A) in response to a suboptimal collagen concentration (0.5 µg/mL). This phenomenon has disappeared at a higher concentration of collagen (5 µg/mL) (Figure 1F).

The platelet activation depends on signalling pathways which are regulated essentially by several isoforms of PKC family. PKC delta isoform is one of the most important members of this family since it is a key

regulator of platelet adhesion, activation, secretion and aggregation [26-29].

The present study shows that PKC delta translocates to the membrane and is phosphorylated on Tyr^{311} in COVID-19 patients. Pre-treatment of platelets with ethanolic extracts of *L. angustifolia* and *R. officinalis* inhibits translocation of PKC delta (Figure 1C) while pre-treatment with all three ethanolic extracts prevents its phosphorylation on Tyr^{311} residue (Figure 1D). No spontaneous aggregation of platelets in the presence of 137 mM NaCl was observed while the complete (the solution in which the platelets are reconstituted/vehicle) showed no effect (Figures 1E,G).

The present work shows that ethanolic extract of medicinal plants can reduce platelet hyper-reactivity observed in COVID-19 patients. These data provide an attractive and promising drug candidate for anti-SARS-CoV-2.

Author contributions

QLA, RL, RH and YZ have designed the study. QLA, RL, RH and YZ have analysed the data. BA, CR and JB performed experiments and helped with data extraction. All the listed authors have read and approved the submitted manuscript.

Funding statement

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Ethics statement

All donors provided written consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Cheikh Zaid Hospital (CEFCZ/PR/2020/PR04)

Conflict-of-interest disclosure

The authors declare that they have no competing financial interests.

Data availability

The data used in this study are included within the article.

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