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Modulation of Coagulation Biomarkers in SARS-CoV2-Induced Mice by Carthamus tinctorius and Dexamethasone Combination Therapy

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ABSTRACT

COVID-19 is a respiratory illness commonly associated with an inflammatory response that extends beyond lung symptoms and impairs blood clotting mechanisms. Hydroxysafflor yellow A, a key bioactive compound isolated from the florets of Carthamus tinctorius, is recognized as a potential therapeutic agent due to its combined anti-inflammatory and anticoagulant actions. Hence, this investigation assessed the influence of ethanol extract from C. tinctorius administered alongside dexamethasone on markers of coagulation in mice challenged with the SARS-CoV-2 spike protein. The florets of Carthamus tinctorius underwent extraction using 98% ethanol. The resulting concentrated extract was applied in the experiment. A total of twenty-five Balb/c mice were involved, with five serving as normal controls and twenty subjected to SARS-CoV-2 induction. These induced animals were then randomly allocated to seven-day regimens including vehicle only, dexamethasone at 2.5 mg/kg body weight, dexamethasone at 2.5 mg/kg body weight plus C. tinctorius extract at 400 mg/kg body weight, or dexamethasone at 2.5 mg/kg body weight plus C. tinctorius extract at 800 mg/kg body weight. Following treatment, analyses were conducted on the pulmonary tissues and blood samples from the mice. Induction with SARS-CoV-2 led to elevations across all evaluated coagulation parameters. Administration of dexamethasone singly or together with C. tinctorius extract at 400 mg/kg body weight produced no decreases in levels of D-dimer, plasminogen activator inhibitor-1, lactate dehydrogenase, platelet-to-leucocyte ratio, or neutrophil-to-leucocyte ratio. Conversely, the combination of dexamethasone and C. tinctorius extract at 800 mg/kg body weight restored normal values for D-dimer, PAI-1, and NLR in the SARS-CoV-2-challenged mice. Combining a higher dosage of C. tinctorius floret extract (800 mg/kg body weight) with dexamethasone provided advantages in alleviating coagulation disturbances linked to SARS-CoV-2..

Keywords: Carthamus tinctorius, COVID-19, Hydroxysafflor yellow A, Inflammation, Thrombosis

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Introduction

Coronavirus disease 2019 (COVID-19) is an infectious condition triggered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Transmission occurs primarily via interpersonal contact and respiratory droplets. Infected individuals often present with symptoms such as fever, headache, and cough [1, 2]. More critical presentations typically involve difficulty breathing stemming from airway inflammation. Apart from inflammatory changes, clotting disruptions—specifically thrombotic events—contribute significantly to illness severity and death rates among COVID-19 cases [3]. Earlier investigations have established links between inflammation and clotting alterations, with strong correlations to clinical worsening in affected patients [4]. Elevated thrombotic risks and coagulation indicators also function as vital predictors of outcome in this population [5-7]. Excessive activation of the renin-angiotensin-aldosterone system (RAAS) has been implicated in inflammatory processes during SARS-CoV-2 infection [8].

Management strategies for COVID-19 vary according to illness severity and phase, incorporating antiviral agents, anti-inflammatory drugs, and supportive treatments [9-11]. Anticoagulant therapy is not part of routine guidelines for COVID-19 management. According to World Health Organization recommendations, dexamethasone remains the primary choice for patients needing respiratory support or mechanical ventilation [9]. That said, various reports indicate that monotherapy with corticosteroids may fall short in addressing COVID-19 challenges. Pairing corticosteroids with anticoagulants could prove advantageous for individuals with moderate-to-severe disease by averting adverse events and boosting recovery prospects [12-14]. Consequently, exploring innovative agents that offer both anti-inflammatory and anticoagulant benefits, when paired with a corticosteroid, could represent an effective therapeutic avenue for COVID-19.

One promising compound is hydroxysafflor yellow A, a major constituent extracted from Carthamus tinctorius Linn., noted for its dual anti-inflammatory and antithrombotic capabilities [15, 16]. Known as safflower, Carthamus tinctorius thrives in arid climates, notably across Southern Asia, China, and India [17]. Extracts from its florets have been applied traditionally and in research as antiviral and inflammation-reducing remedies [15, 17]. Evidence supports diverse pharmacological roles for Carthamus tinctorius, encompassing immune regulation, antioxidation, delay of aging, resistance to low oxygen, reduction of fatigue, suppression of inflammation, prevention of liver fibrosis, tumor inhibition, pain relief, and inhibition of clot formation [18-20]. Experiments using mouse macrophage lines have indicated that extracts from C. tinctorius reduce inflammation through blockade of the NF-κB pathway and enhancement of the Nrf-2/HO-1 pathway [20]. In models of blood stagnation in rats, the extract enhanced blood flow properties and reduced red cell clumping [21]. Furthermore, in mice with chemically induced thrombosis via FeCl3, treatment with C. tinctorius extract exhibited clear antithrombotic activity [22]. Accordingly, this work was designed to explore the impacts of floret extract from Carthamus tinctorius, when used conjointly with dexamethasone, on clotting parameters in a mouse model involving SARS-CoV-2 spike protein induction.

Materials and Methods

Ethical considerations

The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia, under reference number KET-908/UN2.F1/ETIK/PPM.00.02/2021. All procedures involving animals complied with the institutional standards for laboratory animal welfare at the Animal Research Facility, Department of Microbiology, Faculty of Medicine, Universitas Indonesia.

Chemicals

The recombinant SARS-CoV-2 spike S protein antigen (UniProt accession P0DTC2, 138 kDa) was procured from Sigma Aldrich, Singapore (catalogue number AGX819). ELISA assays for quantifying D-dimer (catalogue EM0979) and PAI-1 (catalogue EM1262) were acquired from Finetest, China. The assay kit for lactate dehydrogenase measurement via spectrophotometry was supplied by Randox Laboratories, Ireland (catalogue LD3842). Ethanol, methanol, and other reagents were analytical-grade products from Merck, Germany.

Plant material

Florets of Carthamus tinctorius were harvested from the Bone district in South Sulawesi, Indonesia, in the 2021 blooming season. Species authentication was carried out by the Taxonomic Laboratory, Universitas Negeri Makassar, Indonesia. A reference specimen was archived at the Herbarium Bogoriense, National Research and Innovation Agency, Cibinong, Indonesia (specimen identifier B-573/V/DI.05.07/11/2021).

A quantity of 200 grams of dried, powdered florets was subjected to maceration in 98% ethanol for 24 hours. The extract was filtered to isolate the liquid portion from the solid residue. The marc underwent two additional maceration cycles. Combined filtrates were evaporated to concentrate using a rotary evaporator with a water bath temperature of 55 °C. This condensed extract was subsequently administered to mice challenged with SARS-CoV-2 components.

SARS-CoV-2 induced mice

The study was a controlled experiment employing Balb/c mice between 12 and 15 weeks old, challenged with SARS-CoV-2 spike S protein. Mice were allocated to five groups of six animals each. Twenty subjects received

15 µg of recombinant SARS-CoV-2 spike S protein in 50 µL saline via intratracheal delivery, followed immediately by 100 µL air bolus. The challenge method followed the approach described by Hansur *et al*. [23]. Starting 24 hours after challenge, the twenty affected mice were randomly distributed into four treatment arms for seven consecutive days: placebo vehicle, oral dexamethasone at 2.5 mg/kg body weight, oral dexamethasone at 2.5 mg/kg body weight combined with Carthamus tinctorius (CT) extract at 400 mg/kg body weight, or oral dexamethasone at 2.5 mg/kg body weight combined with CT extract at 800 mg/kg body weight. Euthanasia occurred on day eight, with subsequent collection of pulmonary tissue and blood specimens.

Hematologic analysis

Whole blood specimens were analysed with a veterinary hematology analyser to obtain counts of platelets, lymphocytes, and neutrophils.

Quantification of D-dimer, plasminogen activator inhibitor-1 (PAI-1), and lactate dehydrogenase (LDH)

Plasma concentrations of D-dimer and PAI-1 were measured using enzyme-linked immunosorbent assays; sample absorbance readings were interpolated against calibration curves on an ELISA reader.

Serum lactate dehydrogenase levels were evaluated by spectrophotometry at 365 nm wavelength, adhering strictly to the kit manufacturer's protocol.

Analysis of hydroxysafflor a sample preparation from extract

Dry Carthamus tinctorius extract (1 mg) was solubilised in 5 mL of 50% methanol, passed through a 0.2 µm syringe filter, and centrifuged at 14000 rpm for 5 minutes. A 50 µL portion of this solution was then diluted with 100 µL methanol before introduction into the UPLC apparatus.

Sample preparation from serum

Methanol (1 mL) was added to 250 µL mouse serum, followed by centrifugation at 14000 rpm for 5 minutes at 5 °C. The clear supernatant was decanted into a fresh vessel and dried completely at 55 °C under nitrogen stream in a TurboVap concentrator. The dry residue was reconstituted with 50% methanol. For tandem mass spectrometry detection, 7 µL of the reconstituted sample was loaded onto the UPLC system.

Sample preparation from lung tissues

A 10 mg portion of lung tissue was homogenised in 250 µL of 0.1 M phosphate buffer (pH 7.4). Subsequently, 1 mL of methanol (CH_3OH) was introduced, and the mixture was vortexed for 30 seconds before centrifugation at 14000 rpm for 5 minutes at 5 °C. A 500 µL volume of the resulting supernatant was transferred to a new tube and evaporated to dryness under nitrogen flow at 55 °C using a TurboVap system. The residue was then redissolved in 50% methanol. For MS/MS analysis, a 7 µL volume of this reconstituted sample was injected into the UPLC instrument.

UPLC-MS/MS system for hydroxysafflor yellow a analysis

Levels of hydroxysafflor yellow A in the C. tinctorius extract, mouse serum, and lung tissue were quantified by UPLC-MS/MS, achieving a lower limit of quantification (LLOQ) of 0.20 ng/mL. Separation was performed on a Poroshell™ 120 EC-C18 column (2.7 µm, 4.6 × 50 mm) maintained at room temperature. The mobile phase comprised methanol : 5 mM ammonium acetate (85:15). Electrospray ionisation (ESI) was operated in negative mode, monitoring the transition from m/z parent 611 to m/z daughter 491 **(Figure 1).** Quantitation of hydroxysafflor yellow A in extract, serum, and lung samples was based on interpolation from a standard calibration curve comparing sample responses to known standards. Full method validation for accuracy and precision was completed prior to analysis of experimental samples.

Statistical analysis

Differences among groups were assessed using one-way analysis of variance (ANOVA), with subsequent post-hoc testing via Fisher's Least Significant Difference (LSD) method. A p-value less than 0.05 was considered indicative of statistical significance.

Results and Discussion

Analysis via UPLC-MS/MS revealed that the Carthamus tinctorius flower extract contained 0.63% (w/w) hydroxysafflor yellow A, equivalent to 0.63 mg per 100 mg of extract.

Previous investigations have reported varying yields of hydroxysafflor yellow A from Carthamus tinctorius, depending on extraction solvents and methods, ranging between 0.023% and 14.564% [15, 24, 25]. Using an ethanol-based extraction approach, Zong *et al*. achieved a content of 0.584%, which is very close to the level observed in the present work [26].

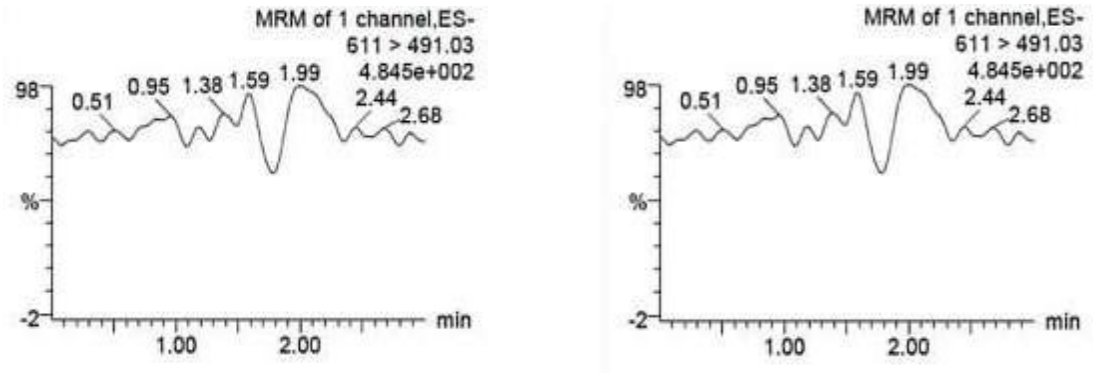
The two doses of Carthamus tinctorius extract administered in this study—400 mg/kg BW and 800 mg/kg BW—corresponded to hydroxysafflor yellow A deliveries of 2.53 mg/kg BW and 5.06 mg/kg BW, respectively.

To date, research on the anticoagulant properties of hydroxysafflor yellow A remains scarce. Existing evidence primarily comes from ex vivo experiments demonstrating beneficial effects on coagulation parameters, with no in vivo data available [27, 28]. One in vivo investigation examined Carthamus tinctorius extract at doses of 100 mg/kg BW and 200 mg/kg BW for improving hemorheological abnormalities in epinephrine-challenged rats [21]. Li *et al*. reported that Carthamus tinctorius treatment could lower blood viscosity and promote better flow characteristics [21]. In the current work, higher doses were selected compared to those used by Li *et al*., based on the assumption that SARS-CoV-2-induced coagulopathy might require greater therapeutic levels than epinephrine-induced models.

D-dimer, PAI-1, and lactate dehydrogenase serve as key indicators for assessing coagulation disturbances in COVID-19 cases [29-31]. Evidence indicates that SARS-CoV-2 frequently alters coagulation cascades, including fibrinolysis [32].

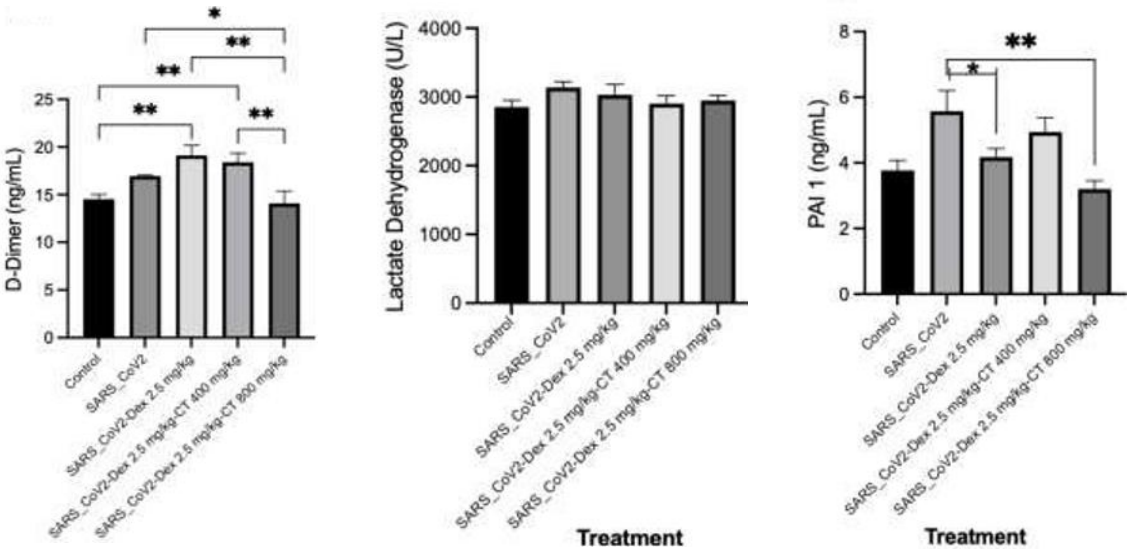
Markedly elevated D-dimer in COVID-19 patients suggests pronounced hyperfibrinolysis driven by plasmin activity. Fibrinolysis represents a vital dynamic process governing fibrin clot formation and breakdown. Under normal conditions, balance is maintained largely by inhibitors such as plasminogen activator inhibitor-1 (PAI-1) and α2-antiplasmin. Disruptions in the ratio of activators to inhibitors can result in pathologies ranging from excessive plasmin-mediated fibrin degradation (hyperfibrinolysis) to impaired fibrinolysis altogether [7, 30, 33].

In this investigation, SARS-CoV-2 induction produced substantial rises in D-dimer and PAI-1 levels, alongside modest elevations in lactate dehydrogenase in the affected mice. Administration of dexamethasone alone or combined with Carthamus tinctorius extract at 400 mg/kg BW failed to restore these markers. In contrast, the higher-dose combination of dexamethasone with C. tinctorius extract at 800 mg/kg BW brought D-dimer, PAI-1, and lactate dehydrogenase back to normal ranges **(Figure 2).** Although lactate dehydrogenase returned to baseline values with the 800 mg/kg BW regimen, the change did not reach statistical significance, attributable to only a minor (~10%) increase in the SARS-CoV-2 control group.



a) b)

Figure 1. Chromatogram displaying (a) blank lung tissue homogenate analysed by UPLC-MS/MS; (b) lung tissue sample spiked with hydroxysafflor yellow A, detected using UPLC-MS/MS with electrospray ionisation (ESI) in negative mode, monitoring the transition from m/z parent 611 to m/z daughter 491.



a) b) c)

Figure 2. Concentrations of (a) D-dimer; (b) lactate dehydrogenase; (c) plasminogen activator inhibitor-1 (PAI-1) in healthy control mice versus SARS-CoV-2-induced mice receiving dexamethasone alone or combined with Carthamus tinctorius (CT) extract; * $p<0.05$; ** $p<0.001$; Dex: dexamethasone 2.5 mg/kg BW; CT 400: Carthamus tinctorius extract 400 mg/kg BW; CT 800: Carthamus tinctorius extract 800 mg/kg BW.

In clinical settings, elevations of LDH by as little as 20% in severe COVID-19 cases have been linked to a up to 6-fold higher risk of complications and death [34, 35]. Here, dexamethasone monotherapy produced only a 3% reduction in LDH. In contrast, co-administration with Carthamus tinctorius extract in SARS-CoV-2-challenged mice achieved an 8–9% decline in LDH. Although dexamethasone is established as standard therapy for moderate-to-severe COVID-19, it failed to lower D-dimer in this model, despite reducing PAI-1. This observation may relate to the timing of corticosteroid intervention during SARS-CoV-2 infection. Research by Rubio *et al*. indicates that early dexamethasone use can promote progression to severe disease, characterised by markedly elevated D-dimer [36]. Therefore, supplementing dexamethasone with Carthamus tinctorius extract at 800 mg/kg BW appears to provide additional benefit by restoring normal D-dimer and PAI-1 levels in SARS-CoV-2-induced mice.

The results also revealed elevations in platelet counts, neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR) following SARS-CoV-2 induction. Neither dexamethasone alone nor its combination with Carthamus tinctorius extract at 400 mg/kg BW or 800 mg/kg BW significantly reduced platelet counts or PLR. However, the higher-dose combination of dexamethasone with C. tinctorius extract at 800 mg/kg BW lowered NLR to near-control levels **(Figure 3).**

SARS-CoV-2 primarily enters via the respiratory tract, targeting the lungs. In the pulmonary compartment, a specialised coagulation process termed bronchoalveolar haemostasis interacts with immune components to contain infection. Acute-phase reactants, including platelets and fibrinogen, are prominently involved and recognised as markers of hypercoagulability [37, 38]. Autopsy studies in COVID-19 patients have confirmed the presence of pulmonary microthrombi [39]. Distinctive respiratory features in COVID-19 suggest that microthrombi contribute substantially to clinical deterioration [40]. In mild cases, strong local fibrinolysis degrades these microthrombi, preserving gas exchange and manifesting as raised D-dimer. Severe disease features exaggerated activation of this pulmonary coagulation cascade [32].

Infected individuals typically exhibit elevated platelet counts, particularly early in the course. Thrombocytopenia is rare in SARS-CoV-2 patients, even among the critically ill [37]. Clinical data show that higher platelet counts correlate with prolonged hospitalisation and poorer prognosis [41]. This rise may stem from cytokine storm, although exact pathways remain unclear [42].

In the current experiment, SARS-CoV-2 induction increased platelet counts in mice, and neither dexamethasone nor its combinations with Carthamus tinctorius extract at either dose reversed this elevation. Notably, the observed

increases did not reach extreme thrombocytosis levels that could precipitate further coagulopathy or overt thrombosis.

Previous ex vivo investigations using rabbit plasma in a non-infectious setup have shown that hydroxysafflor yellow A, the primary bioactive constituent of C. tinctorius, exerts anticoagulant activity by suppressing platelet aggregation, preventing thrombus development, and decreasing blood viscosity [27, 28].

Beyond platelet counts, the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) have recently emerged as reliable indicators of systemic inflammation in the circulation [43]. Disruptions in neutrophil and lymphocyte balances are frequently seen in progressive stages of COVID-19, potentially linked to cytokine storm phenomena where neutrophils act as key effector cells. The NLR effectively captures this neutrophil-lymphocyte relationship and may function as an early predictor of severe disease trajectory [44, 45]. Similarly, elevated PLR serves as a marker of pro-inflammatory status and a prognostic indicator for COVID-19 advancement, with multiple reports examining its association with diagnosis and mortality in affected patients [45, 46].

In COVID-19 management, higher dexamethasone doses are required to curb overwhelming systemic inflammation through suppression of neutrophil activity and prevention of neutrophil accumulation in the lungs and other affected organs [47]. Evidence suggests that lower dexamethasone doses provide varying degrees of anti-inflammatory protection in COVID-19 [48]. In the present work, only the combination of dexamethasone with the higher dose of C. tinctorius extract (800 mg/kg BW) effectively reduced NLR, aligning with the observed improvements in D-dimer and PAI-1.

Treatment with Carthamus tinctorius extract at 400 mg/kg BW and 800 mg/kg BW alongside dexamethasone yielded lung tissue concentrations of 0.353 ng hydroxysafflor yellow A per 100 mg tissue and 0.638 ng per 100 mg tissue, respectively **(Figure 4).** However, hydroxysafflor yellow A remained undetectable in plasma.

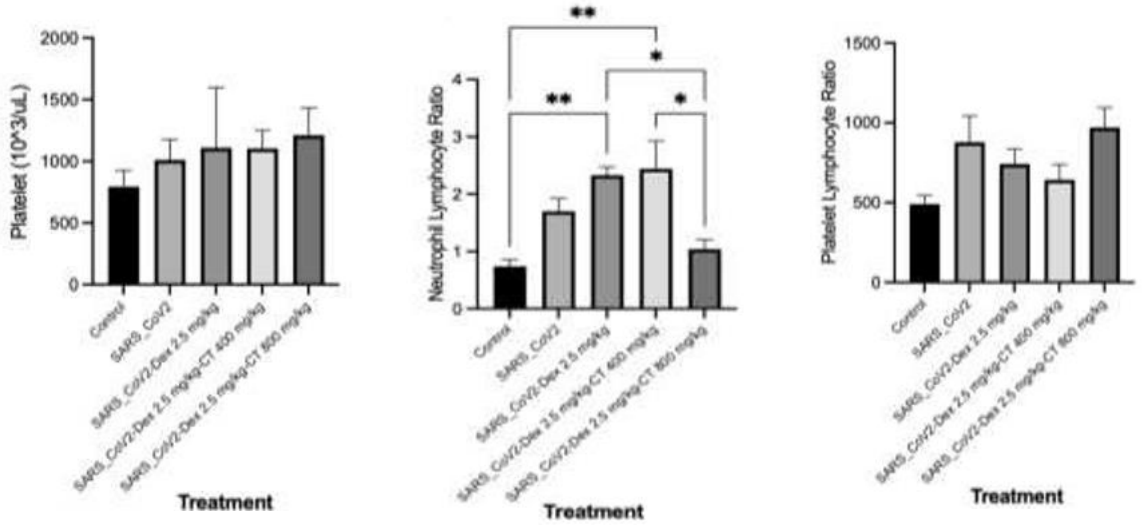
Hydroxysafflor yellow A belongs to Biopharmaceutics Classification System (BCS) class III, characterised by poor oral bioavailability [15]. A prior investigation in normal and diabetic cardiomyopathy mouse models reported peak plasma concentrations (Cmax) of 2.41 µg/mL and 4.08 µg/mL following 60 mg/kg BW dosing [49]. By comparison, in this study, samples collected 24 hours after seven consecutive days of Carthamus tinctorius administration at 400 mg/kg BW and 800 mg/kg BW (equivalent to 2.53 mg/kg BW and 5.06 mg/kg BW hydroxysafflor yellow A) revealed measurable levels in lung tissue but none in plasma. Nonetheless, these modest pulmonary concentrations proved sufficient to mitigate elevations in coagulation markers in SARS-CoV-2-induced mice.

Pharmacokinetic data from intravenous hydroxysafflor yellow A in rats indicated a distribution volume of 0.29 L/kg and a half-life of 0.83 hours (approximately 50 minutes), translating to an overall distribution volume of 1.16 L in a 250-gram rat [50]. Rat total blood volume is roughly 64 mL/kg (or 16 mL in a 250-gram animal) [51]. Thus, the large distribution volume (1160 mL) combined with rapid elimination explains the absence of detectable hydroxysafflor yellow A in plasma 24 hours post-final dose, reflecting swift tissue uptake. The current findings confirm substantial distribution to lung tissue.

While hydroxysafflor yellow A represents the dominant compound in Carthamus tinctorius extract, the florets contain numerous other constituents, including alkaloids, flavonoids, lignans, organic acids, and polyacetylenes [52].

Collectively, these safflower components contribute to the observed anti-inflammatory and anticoagulant effects in the SARS-CoV-2-induced mouse model.

The present data indicate that additional research on C. tinctorius flower extracts could help reduce coagulopathy risks in SARS-CoV-2 infection. More preclinical and clinical investigations are warranted to elucidate mechanisms, identify key active principles, and optimise dosing for positioning Carthamus tinctorius extracts as a potential adjunct to dexamethasone in managing SARS-CoV-2-related coagulation disturbances.



a) b) c)

Figure 3. (a) Platelet count; (b) neutrophil-to-lymphocyte ratio (NLR); (c) platelet-to-lymphocyte ratio (PLR) in healthy control mice or SARS-CoV-2-induced mice treated with dexamethasone alone or combined with Carthamus tinctorius (CT) extract; * p<0.05; ** p<0.001; Dex: dexamethasone 2.5 mg/kg BW; CT 400: Carthamus tinctorius extract 400 mg/kg BW; CT 800: Carthamus tinctorius extract 800 mg/kg BW.

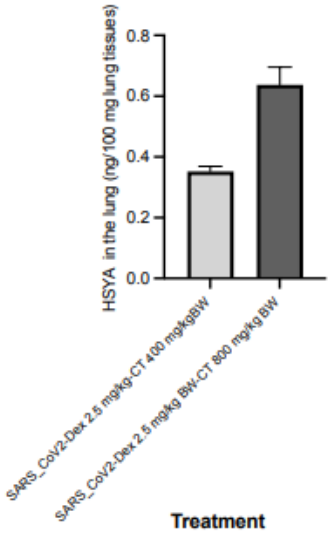


Figure 4. Levels of hydroxysafflor yellow A (HSYA) in lung tissue from SARS-CoV-2-induced mice following treatment with dexamethasone plus Carthamus tinctorius extract at 400 mg/kg BW or 800 mg/kg BW. Dex: dexamethasone 2.5 mg/kg BW; CT 400: Carthamus tinctorius extract 400 mg/kg BW; CT 800: Carthamus tinctorius extract 800 mg/kg BW.

Conclusion

Combining a high dose of C. tinctorius flower extract (800 mg/kg BW) with dexamethasone effectively attenuates coagulation abnormalities in SARS-CoV-2-induced mice. This benefit is largely attributable to the anti-inflammatory action of its key constituent, hydroxysafflor yellow A, within pulmonary tissues.

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Conflict of Interest: None

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Ethics Statement: None

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