



Research article

Wild-type SARS-CoV-2–ACE2 binding inhibitory activity of Zingiberaceae herbs and spices

Rungnapa Boonpawa^{1,*} and Surassawadee Promyou²

¹ Department of Food Technology and Nutrition, Faculty of Natural Resources and Agro-industry, Kasetsart University Chalemphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, 47000, Thailand

² Department of Agriculture and Resources, Faculty of Natural Resources and Agro-industry, Kasetsart University Chalemphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, 47000, Thailand

* **Correspondence:** Email: rungnapa.boon@ku.th; Tel: +66(0)631468747.

Abstract: COVID-19 is a disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged in late December 2019. The present study assessed the potential of herbs and spices from the Zingiberaceae family to inhibit binding of the wild-type SAR-CoV-2 spike protein to human angiotensin-converting enzyme 2 (ACE2) using an in vitro binding inhibition assay. From the 21 assessed Zingiberaceae extracts, 10 significantly inhibited binding at 200 µg/mL. The binding inhibitory potential of the extracts did not correlate directly with total phenolic and flavonoid content, but rather with specific phytochemicals (active compounds). Among the tested extracts, *B. rotunda* extracts from three different sources exhibited significant inhibitory activity (42%–46% non-inhibition). Panduratin A (identified compound) and 4-hydroxypanduratin A (tentatively identified compound) were identified as active compounds responsible for the binding inhibitory activity in *B. rotunda* extracts. The inhibitory dose-response curve for binding inhibition was obtained for panduratin A, yielding an IC₅₀ of 0.38 µg/mL. Overall, Zingiberaceae plants, especially *A. biflorum*, *B. rotunda*, *C. aromatic*, *C. mangga*, *C. zedoaria*, *K. parviflora*, *Kaempferia* sp., and *Z. montanum*, exhibited potent binding inhibitory activity against wild-type SARS-CoV-2 cellular entry. Although this finding indicates the potential of Zingiberaceae plants as anti-SAR-CoV-2 agents, further research is needed, as the present study was limited to a biochemical assay using the wild-type spike protein. Thus, further validation using clinically relevant SARS-CoV-2 variants, cell-based viral infection models, and in vivo studies is required before antiviral efficacy can be concluded.

Keywords: bioactive compound; Coronavirus; COVID-19; Panduratin A; SAR-CoV-2 cellular entry

List of Abbreviations

ACE2: Angiotensin-converting enzyme 2

CE: Catechin equivalent

COVID-19: Coronavirus disease-2019

DW: Dry weight

GAE: Gallic acid equivalent

PAE: Panduratin A equivalent

RBD: Receptor-binding domain

SAR-CoV-2: Severe acute respiratory syndrome coronavirus 2

1. Introduction

Since the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in late December 2019, this coronavirus has continued to pose a significant threat to public health worldwide, resulting in 779 million cumulative cases and over 7 million deaths (WHO, data as of December 24, 2025; <https://covid19.who.int/>). SARS-CoV-2 shares roughly 80% sequence identity of the spike protein with SARS-CoV-1 and MERS-CoV (Middle East respiratory syndrome coronavirus), the previous coronavirus outbreak strains in 2002–2004 and 2012, respectively [1,2]. COVID-19 is a disease caused by SARS-CoV-2, displaying various symptoms depending on the host's health status, ranging from no symptoms to life-threatening pneumonia and cytokine-related syndromes causing multi-organ failure [3].

Similar to the previous outbreak strains, the cellular entry mechanism of SARS-CoV-2 critically involves binding of the receptor-binding domain (RBD) of the spike protein to angiotensin-converting enzyme 2 (ACE2) receptor [2,4]. ACE2 receptors are expressed on the surfaces of many human cells, including the lungs, kidneys, liver, brain, heart, and vessels [3]. Since the binding of the spike protein to ACE2 is crucial for cellular entry, viral replication, and infection, it is therefore a promising target for preventive and therapeutic interventions for COVID-19 [5,6].

The currently used drugs to treat COVID-19 (e.g., remdesivir, paxlovid, and molnupiravir) are reported to have various side effects, such as nausea, diarrhea, impaired taste, dizziness, elevated liver transaminase and prothrombin, and induced hypersensitivity reactions [7]. Due to the side effects, several studies have shifted toward multifactorial, low-side-effect, more affordable, and more readily available natural compounds rather than synthetic drugs. Natural botanical extracts and phytochemicals have received considerable attention as potential alternatives for the prevention and treatment of COVID-19 [8]. If proven effective, the use of natural botanical extracts and phytochemicals could be more affordable for the public, especially in poorer regions. Polyphenols are secondary metabolites abundantly present in plants, exhibiting broad antiviral activity against various groups of viruses and exerting other health-beneficial effects such as anti-inflammatory and immunomodulatory properties [9,10]. Based on in silico and in vitro evidence, the underlying mechanism of polyphenols against SARS-CoV-2 might involve binding to the spike protein or ACE2 on the host cell membrane, hampering viral cellular entry, as well as binding to viral proteases, inhibiting viral replication in the host cell [10,11]. Moreover, polyphenols, as potent anti-inflammatory compounds, would be beneficial in COVID-19 treatment in inhibiting the cytokine-driven

hyperinflammatory syndrome caused by SARS-CoV-2. A recent study using pharmacoinformation approaches found that among the 20 tested phytochemicals and four approved anti-SARS-CoV-2 drugs (i.e., remdesivir, favipiravir, hydroxychloroquine, and ivermectin), only betulinic acid, β -sitosterol, and ivermectin displayed high binding affinities to the SARS-CoV-2 spike RBD protein of five wild-type and four variants (i.e., Alpha, Beta, Delta, and Omicron) [12]. Another important aspect that remains to be investigated is the relationship between the total phytochemical content and SARS-CoV-2–ACE2 binding inhibitory activity. There is inconclusive evidence regarding the relationship between phytochemical contents (e.g., total phenolic and flavonoid content) and the biological activity of plant extracts. For example, some studies have revealed a strong correlation between total phytochemical content and biological activity, especially antioxidant activity [13,14], whereas another study reported a weak-to-moderate correlation between total phenolic content and antioxidant, anti-neuraminidase, and anti-cyclooxygenase-2 activities [15]. Nevertheless, this knowledge is important to use as a primary justifying criterion for selecting natural botanical extracts of interest for continued investigation of their potential anti-SARS-CoV-2 activity.

Herbs and spices from the Zingiberaceae family have a long history of use as traditional medicines in several countries [16]. According to ethnobotanical documentation, during the COVID-19 pandemic between 2020 and 2022, medicinal plants belonging to Zingiberaceae were the most used to manage COVID-19 in Asia and Africa [8,17,18]. Well-known plants in this family include ginger (*Zingiber officinale* R.), turmeric (*Curcuma longa* L.), and fingerroot [*Boesenbergia rotunda* (L.) Mansf.]. These plants contain a wide variety of phytochemicals that exhibit biological activities, including antimicrobial, antioxidant, antiviral, and anti-inflammatory effects [16,19,20]. Current findings suggest that Zingiberaceae plants have potential as anti-SARS-CoV-2 agents. A molecular docking study found that curcumin, a major bioactive compound in *C. longa*, showed strong binding affinity for the host-specific receptors furin and ACE2, while gingerol, a major bioactive compound in *Z. officinale*, showed strong binding affinity for the SARS-CoV-2 spike protein [21]. Kanjanasirirat et al. [22] found that *B. rotunda* and its major bioactive compound, panduratin A, displayed potent inhibitory activity against SARS-CoV-2 viral entry and replication in Vero E6 and Calu-3 cell lines. A bioactivity-guided approach was adopted because the phytochemical composition of crude botanical extracts does not necessarily predict their biological activity. Therefore, extracts were first screened for SARS-CoV-2 spike protein–ACE2 binding inhibitory activity, followed by phytochemical analysis to examine the relationship between phytochemical content and observed activity. The most active extract was subsequently selected for compound identification and inhibitory dose-response analysis to determine the active constituents and their potency. Although the antiviral activity of *B. rotunda* and panduratin A has previously been demonstrated in cell-based and antiviral assays, comparative information on the SARS-CoV-2 spike protein–ACE2 binding inhibitory activity among Zingiberaceae species and the relationship between phytochemical contents and binding inhibition remains limited.

Therefore, the present study aimed to investigate the inhibitory activity of Zingiberaceae plants, focusing on the viral entry mechanism of SARS-CoV-2–ACE2 interaction. Furthermore, the correlation between the inhibitory activity and the total phytochemical content (total phenolic and flavonoid contents) of the plant extracts was also investigated. Subsequently, the extract that exerted the highest inhibitory activity was selected for phytochemical composition analysis, fractionation, and inhibitory activity of the isolated compounds. Plants in the family Zingiberaceae were selected for study because they are widely cultivated and commonly used as herbs and spices worldwide.

2. Materials and methods

2.1. Materials

Human ACE2 (His-Avi-Tag, Biotin-labeled), immunobuffer, HRP colorimetric substrate, blocking buffer 2, SARS-CoV-2-spike S1 RBD wild-type (319-451-Avi-His), and streptavidin-HRP were purchased from BPS Bioscience (San Diego, USA). Dimethyl sulfoxide (DMSO) and Folin–Ciocalteu were obtained from LOBA Chemie (Mumbai, India). Ethanol (95% v/v, industrial grade) was purchased from Chemipan Corporation (Rayong, Thailand). Acetonitrile (HPLC grade) and ultrapure water (HPLC grade) were obtained from RCI Labscan (Bangkok, Thailand). Sodium nitrite (NaNO_2) and aluminum chloride (AlCl_3) were obtained from Kemaus (N.S.W, Australia). Sodium hydroxide (NaOH) and sodium carbonate (Na_2CO_3) were obtained from QReC (New Zealand). Gallic acid, (+)-catechin, and quercetin were purchased from Sigma-Aldrich (Missouri, USA). Panduratin A (80.89% purity) was obtained from Toronto Research Chemicals (Canada). Phosphate-buffered saline, pH 7.4 (PBS), was purchased from Gibco (Paisley, UK).

2.2. Plant extract preparation

Zingiberaceae plants were collected from three regions in Northeastern, Northern, and Central Thailand during June–December 2022. The plants were authenticated at the Faculty of Natural Resources and Agro-industry, Kasetsart Chalermphrakiat Sakon Nakhon Province Campus. Fresh specimens were kept in the faculty greenhouse. Fresh samples were dried at 50 °C before being ground into powder. The Zingiberaceae plants used are listed in Table S1 (Supplementary material).

Plant extracts were prepared by extracting 5 g of plant powder twice with 25 mL of ethanol (95% v/v) with continuous shaking at 37 °C for 24 h. The pooled extracts were filtered through filter paper (Whatman no. 1) and dried under vacuum using a rotary vacuum evaporator (Hei-VAP Expert, Heidolph, Germany). The dried crude extracts were dissolved in DMSO and stored at -20 °C until further analysis.

2.3. Total phenolic content analysis

The Folin–Ciocalteu method was carried out to determine total phenolic content following the methods described by Attard [23] with some modifications. Briefly, mixtures consisting of 10 μL of extracts (1 mg/mL) and 100 μL of Folin–Ciocalteu reagent were freshly prepared and rested at room temperature for 5 min prior to adding 80 μL of Na_2CO_3 (1 M). Mixtures were incubated and continuously shaken in the dark at 37 °C for 40 min, after which the absorbance was measured at 750 nm using a Synergy HT (BioTek, USA). The total phenolic content was estimated and expressed as milligrams of gallic acid equivalent per gram of dried plant weight (mg GAE/g DW). The blank control was performed by adding 10 μL of DMSO.

2.4. Total flavonoid content analysis

Total flavonoid content was analyzed using the NaNO_2 – AlCl_3 method based on Herald et al. [24] with some modifications. Mixtures consisting of 25 μL of extracts (1 mg/mL), 100 μL of water, and 10 μL of NaNO_2 (50 g/L) were prepared and rested at room temperature for 5 min before adding 15 μL of AlCl_3 (100 g/L). After 6 min, 100 μL of NaOH (1 M) was added, followed by immediately adding 50 μL of water. The mixtures were thoroughly mixed before measuring absorbance at 510 nm using a

Synergy HT (BioTek, USA). The total flavonoid contents were estimated and expressed as milligrams of catechin equivalent per gram of dried plant weight (mg CE/g DW). The blank control was performed by adding 25 μ L of DMSO.

2.5. HPLC determination and fractionation of *B. rotunda* extracts

Based on the binding inhibitory activity of Zingiberaceae extracts (see section 3.1), all three *B. rotunda* extracts were selected for further analysis using the HPLC-DAD (1200 series) connected with a guard column XBD-C18 (4.6×12.5 mm, 5 μ M), and a ZORBAX Eclipse C18 column (4.6×150 mm, 5 μ M) manufactured by Agilent (Germany). The HPLC methods were developed according to Adhikari et al. [25] with some modifications. The flow rate, column temperature, and injection volume were set to 1 mL/min, 40 $^{\circ}$ C, and 20 μ L, respectively. All samples were filtered with a PTFE membrane filter (0.45 μ m) prior to injection. The mobile phases consisted of 0.1% formic acid (A) and acetonitrile (B). The gradient started from 20% to 59.5% B (0–25 min), 59.5% B (25–30 min), 59.5%–100% B (30–34 min), 100% B (34–44 min), 100%–20% B (44–48 min), and 20% B (48–53 min). Major bioactive compounds were quantified and expressed as milligrams of panduratin A (mg PAE) per gram, integrating at 290 nm.

To further assess the key bioactive compound in *B. rotunda* extracts responsible for the inhibitory activity against SARS-CoV-2–ACE2 binding, the major bioactive compounds in the extracts were fractionated using the HPLC-DAD methods described above. The isolated fractions were collected and pooled from 10 injections of 5 mg/mL extracts. The mobile phases were removed using a rotary vacuum evaporator (Hei-VAP Expert, Heidolph, Germany). The obtained dryness was redissolved in 0.5 mL of DMSO and stored at -20 $^{\circ}$ C until further analysis.

2.6. SARS-CoV-2 spike protein–ACE2 binding inhibition assay

The binding inhibitory activity of Zingiberaceae extracts was determined using an in vitro SARS-CoV-2 spike S1 RBD-ACE2 binding inhibition assay modified from methods described by BPS Bioscience Spike S1 RBD (SARS-CoV-2)-ACE2 Inhibitor Screening Colorimetric Assay kit (catalog no. 78018). Wild-type SARS-CoV-2 spike proteins (1.5 μ g/mL) were freshly prepared in PBS. Spike proteins (50 μ L) were then bound to the cooled 96-well plate and kept at 4 $^{\circ}$ C overnight. The plate was washed three times with 100 μ L of the immune buffer (i.e., the washing step). Prior to incubation for 1 h, 100 μ L of blocking buffer 2 was added. The bound plate was then washed before adding the reaction mixture. The reaction mixture with a total volume of 60 μ L was prepared by adding 36 μ L of blocking buffer 2, 12 μ L of plant extracts or quercetin (from a 40 $\times$ stock dilution in blocking buffer 2), and 12 μ L of ACE2 (8 ng/ μ L prepared in blocking buffer 2). Quercetin was previously reported to inhibit binding between the SARS-CoV-2 spike protein and ACE2 [21,26] and was thus used as a positive control in the present study. The reactions were initiated by adding 50 μ L of the reaction mixture to the bound plate, incubating at room temperature for 1.5 h, and then applying the washing step. To generate the color, 100 μ L of streptavidin-HRP (diluted 1000 $\times$ in blocking buffer 2) was added and incubated at room temperature for 1 h, followed by the washing step. Colorimetric HRP (100 μ L) was subsequently added and incubated for 5 min before adding 100 μ L of HCl (1 N). The absorbance was measured immediately at 450 nm using a microplate reader (Synergy HT, BioTek Instruments, USA). Blank control was performed without ACE2 and inhibitor, while non-inhibition (solvent control) was performed with DMSO (40 $\times$ dilution of DMSO in blocking buffer 2). During the reactions, DMSO in all samples was kept at 0.5% as suggested by the manufacturer, to be <1% to

avoid interference with binding. The binding activity between wild-type SARS-CoV-2 spike protein and ACE2 was linearly increased at ACE2 concentrations of 1–10 ng/μL (data not shown). The binding activity was expressed as % non-inhibition:

$$\% \text{non-inhibition} = \frac{(Abs.in - Abs.blank)}{(Abs.non - Abs.blank)} \times 100 \quad (1)$$

where Abs is the absorbance reading at 450 nm for the inhibitor (in), blank, and non-inhibition (non).

2.7. Inhibitory dose-response curve of *B. rotunda* phytochemicals on SARS-CoV-2 spike protein–ACE2 binding

The isolated fractions from *B. rotunda* extracts were assessed for their inhibitory activity against SARS-CoV-2–ACE2 binding, using the methods described in Section 2.6. The isolated fraction with the highest binding inhibitory activity was then subjected to further analysis to obtain the inhibition dose-response curve. The IC₅₀ value was obtained by fitting the data to the standard four-parameter dose-response curves with the steepness (HillSlope) constrained to -1, using GraphPad Prism version 10.1.1 (GraphPad Software, San Diego, California, USA). The four-parameter dose-response curve inhibition equation is as follows:

$$Y = Bottom + \left(\frac{Top - Bottom}{1 + 10^{(logIC_{50} - X)HillSlope}} \right) \quad (2)$$

2.8. Statistical analysis

Statistical analysis was carried out using SPSS version 26.0.0.0 (IBM, USA). A t-test was performed to assess whether there was a significant difference in wild-type SARS-CoV-2 spike protein–ACE2 binding activity between a non-inhibited control and a tested inhibitor. One-way ANOVA was used to assess differences in phytochemical content among Zingiberaceae plant extracts, followed by Duncan's multiple range test for post hoc multiple comparisons. Pearson correlation was used to assess the relationship between binding inhibitory activity and phytochemical content in the extracts. A significance level of $P \leq 0.05$ was set.

3. Results

3.1. Inhibitory activity of Zingiberaceae extracts on wild-type SARS-CoV-2 spike protein–ACE2 binding

The inhibitory activity of Zingiberaceae extracts at a final concentration of 200 μg/mL against wild-type SARS-CoV-2 spike protein–ACE2 binding is shown in Figure 1. Compared with the non-inhibited control, 10 of 21 extracts significantly inhibited binding. Among those, all three *B. rotunda* rhizome extracts significantly inhibited binding by 42%–46%. Other extracts that showed significant inhibition were *A. biflorum*, *C. aromatic*, *C. mangga*, *C. zedoaria*, *K. parviflora*, *Kaempferia* sp., and *Z. montanum*, with inhibition ranging from 30% to 41%. Quercetin was used as a positive control in the present study, as previously shown to have high binding affinity for the SARS-CoV-2 spike RBD protein in a molecular docking study [21] and to be the most potent human ACE2 inhibitor among the tested flavonoids, with an IC₅₀ of 4.48 μM [26]. According to the results, quercetin at a concentration of 6.62 μM could inhibit binding by about 68%, confirming the validity of the currently applied in

vitro SARS-CoV-2 spike protein–ACE2 binding inhibition assay.

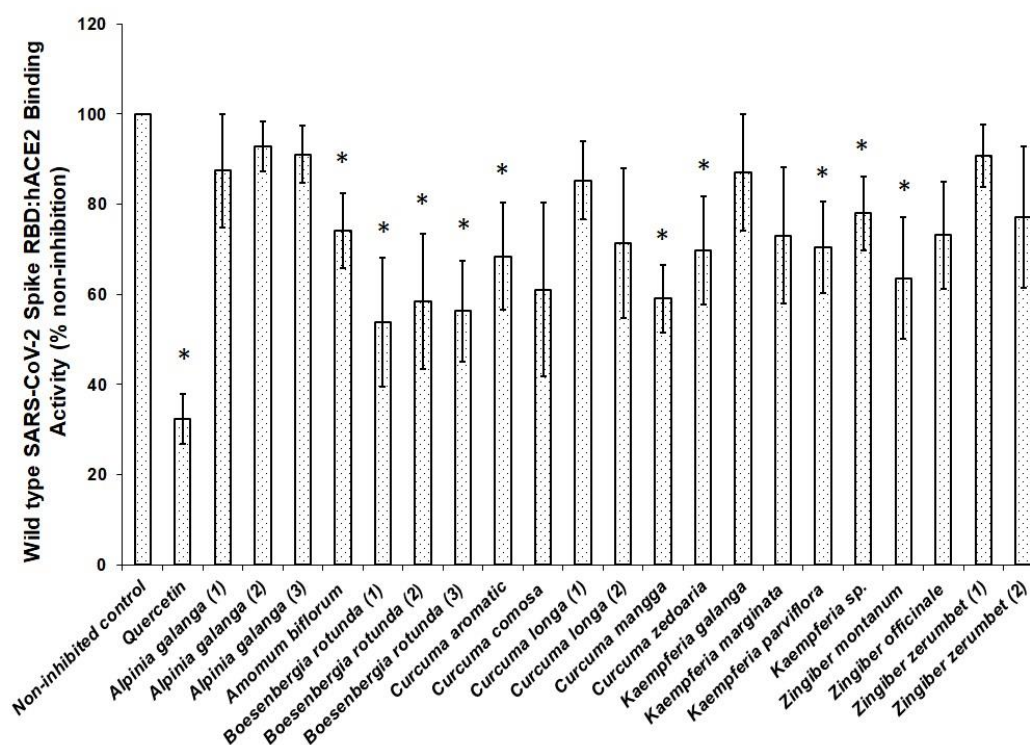


Figure 1. Wild-type SARS-CoV-2-Spike RBD–ACE2 binding activity in the presence of quercetin (2 $\mu\text{g/mL}$ or 6.62 μM) and ethanolic Zingiberaceae extracts (200 $\mu\text{g/mL}$). * Represents significant differences ($P \leq 0.05$) between a non-inhibited control and a tested inhibitor, determined by t-test. Numbers in brackets represent the different sources of the plant sample.

3.2. Phytochemical contents in Zingiberaceae extracts and correlation with the binding inhibitory activity

The total phenolic contents of Zingiberaceae extracts, as shown in Figure 2(A), ranged from 0.53 to 28 mg GAE/g DW plant. The extracts of *C. aromatica*, *C. longa* (2), and *C. zedoaria* contained the highest content. The lowest phenolic contents were observed in the extracts of *A. biflorum*. For the total flavonoid contents, as depicted in Figure 2(B), *C. longa* (2) contained the highest content, followed by *C. zedoaria* and *C. aromatica*, while *A. biflorum*, *K. galanga*, and *Kaempferia* sp. contained the least amount of total flavonoids. Total flavonoid contents of the extracts ranged from 0.18 to 23 mg CE/g DW plant.

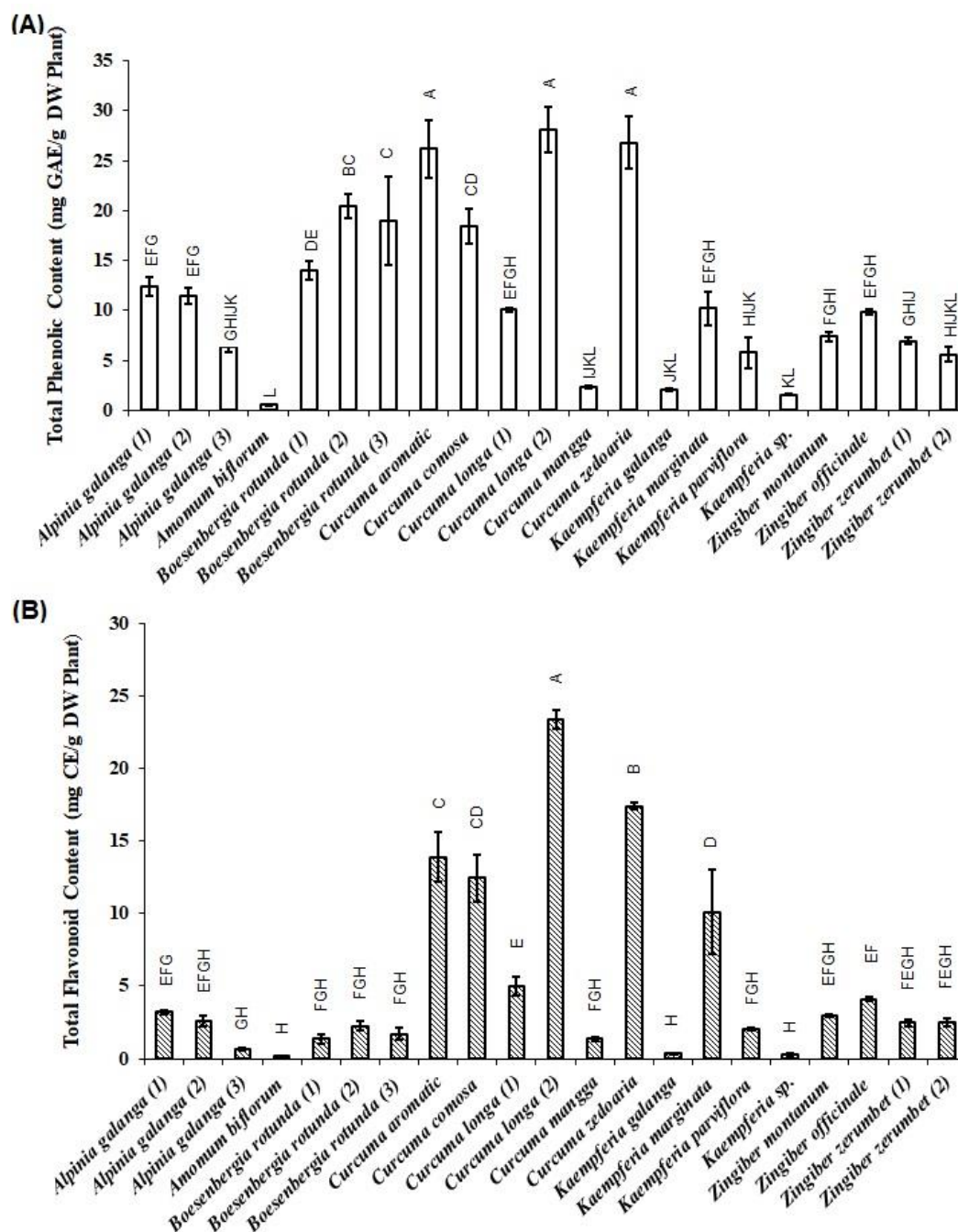


Figure 2. Phytochemical contents of ethanolic Zingiberaceae extracts: (A) Total phenolic contents expressed as mg gallic acid equivalent/g dried weight plant and (B) total flavonoid contents expressed as mg catechin equivalent/g dried weight plant. Different letters represent a significant difference at $P \leq 0.05$, determined by Duncan's multiple range test. Numbers in brackets represent different sources of the plant sample.

Pearson's correlation coefficients were estimated to assess the relationship between phytochemical contents and the inhibitory activity against wild-type SARS-CoV-2 spike protein–ACE2 binding of Zingiberaceae extracts. A moderate correlation was observed between the binding inhibitory activity and the total phenolic content ($r = 0.364$), whereas a low correlation was observed for the total flavonoid content ($r = 0.169$). Thus, the binding inhibitory activity observed in the present

study is likely attributable to specific phytochemicals (active compounds) rather than total phenolic or flavonoid contents.

3.3. Phytochemical compositions and wild-type SARS-CoV-2 spike protein–ACE2 binding inhibitory activity in *B. rotunda* extracts

B. rotunda extracts were selected as the plant of interest for further experiments because all three extracts from different sources significantly inhibited binding. The chromatograms, as shown in Figure S1(A),(B) in the Supplementary material, reveal that all three extracts contain six major compounds. However, the composition of these compounds differs across the three extracts (Table 1). The amount of these compounds was estimated based on the standard compound panduratin A, which integrates at 290 nm (see Figure S2 in the Supplementary material).

Table 1. Amount of six major phytochemicals in *Boesenbergia rotunda* extracts.

<i>B. rotunda</i>	Phytochemical content (mg PAE/g extract) ^a					
	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V	Fraction VI
1	6.65±0.02 ^b	23.89±0.63 ^b	52.80±3.64 ^b	130.45±4.74 ^b	28.06±5.03 ^{ab}	81.01±3.09 ^a
2	17.59±0.84 ^a	37.45±4.83 ^a	86.79±5.79 ^a	78.30±4.59 ^c	38.12±3.60 ^a	69.89±3.94 ^b
3	7.25±0.27 ^b	32.69±1.98 ^{ab}	74.01±1.54 ^{ab}	155.58±7.39 ^a	21.78±3.25 ^b	72.65±1.69 ^{ab}

*Note: ^aData expressed as average ± standard error from triplicate experiments. All data expressed as mg panduratin A equivalent/g extract (mg PAE/g extract). Panduratin A calibration curve integrated at 290 nm: $Y = 88.743X - 366.81$, $R^2 = 0.9901$. Different letters in the vertical direction represent a significant difference at $P \leq 0.05$, determined by Duncan's multiple range test.

All six compounds were isolated and tested for inhibitory activity against wild-type SARS-CoV-2 spike protein–ACE2 binding. The results show that Fractions IV, V, and VI significantly inhibit binding compared with the non-inhibition control, as shown in Figure 3(A). Fractions V and VI exhibit the highest inhibitory activity of about 60%. Fraction VI co-eluted with a standard compound, panduratin A, and was thus identified as panduratin A. Fraction V has a UV spectrum ($\lambda_{\max}$ at 290 nm) similar to that of panduratin A, as also observed by Adhikari et al. [25] and Tan et al. [27], suggesting a conjugated ketone moiety, and was tentatively identified as 4-hydroxypanduratin A. Due to the limited amounts of Fractions V and VI and the availability of the standard compounds, panduratin A was used to determine the inhibitory dose-response curve against the SARS-CoV-2 spike protein–ACE2 binding (Figure 3(B)). The data showed a good fit to the standard four-parameter inhibition dose-response curves, with a goodness-of-fit (R-squared) of 0.9525. The IC_{50} value for the binding inhibitory activity of panduratin A was estimated to be 0.38 $\mu\text{g/mL}$ (0.9 μM).

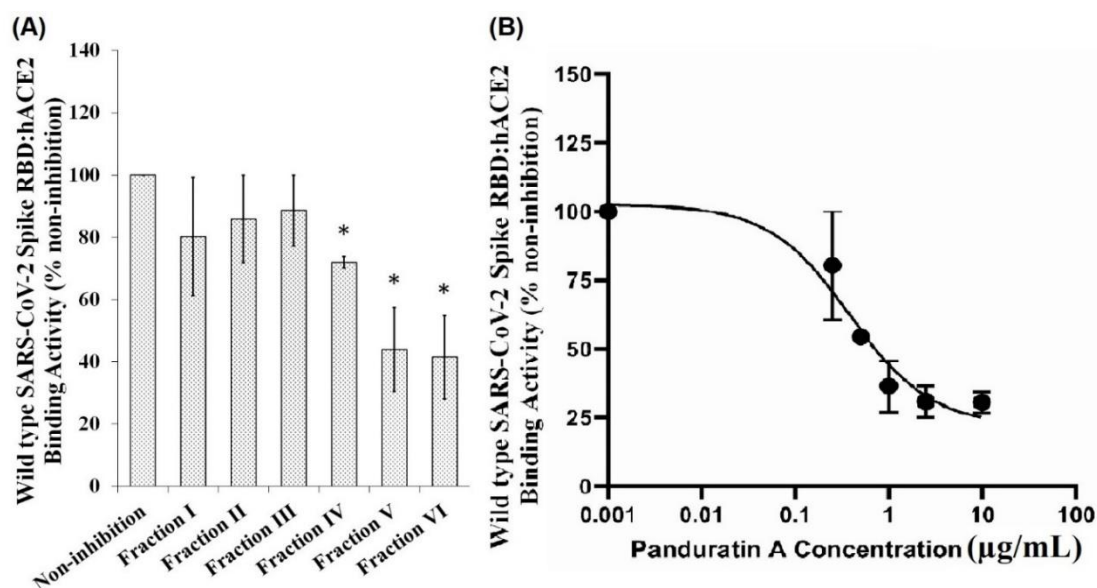


Figure 3. Inhibitory activity of (A) isolated bioactive compounds from the *Boesenbergia rotunda* rhizome extracts and (B) the inhibitory dose-response curve of panduratin A against wild-type SARS-CoV-2 Spike RBD–ACE2 binding. Data are expressed as mean $\pm$ standard error from triplicate experiments. * Represents significant differences ($P \leq 0.05$) between a non-inhibited control and a tested inhibitor, determined by t-test.

4. Discussion

Considering the adverse effects of currently used drugs and the need for more affordable COVID-19 treatments, exploring botanical extracts and phytochemicals for prevention and therapy is of interest. This study aimed to evaluate the potential of Zingiberaceae plants to inhibit the binding of the wild-type SARS-CoV-2 spike protein to human ACE2. The interaction between the viral spike protein and the host ACE2 receptor is a crucial step for viral entry into cells. In this study, quercetin was used as a positive control in the in vitro binding inhibition assay (Figure 1). Previous research showed that quercetin has a high binding affinity to the SARS-CoV-2 spike RBD protein based on molecular docking studies and was the most effective human ACE2 inhibitor among the flavonoids tested, with an IC₅₀ of 4.48 μ M. Similarly, our results indicate that quercetin at 6.62 μ M can inhibit binding by approximately 68%, supporting its potential as a SARS-CoV-2 inhibitor. Importantly, the positive control results confirmed the validity of the current in vitro SARS-CoV-2 spike protein–ACE2 binding inhibition assay.

Ten out of twenty-one Zingiberaceae extracts, at a concentration of 200 μ g/mL, significantly inhibited the interaction between the SARS-CoV-2 spike protein and ACE2 compared with the non-inhibited control. The binding inhibitory activity might be due to polyphenols, an important group of phytochemicals abundantly found in plants. Polyphenols have previously been shown to have a potential inhibitory effect on SARS-CoV-2 cellular entry [26,28–30]. The present study determined the total phenolic and flavanol contents as well as their correlation with SARS-CoV-2 spike protein–ACE2 binding inhibitory activity, as these parameters may serve as indicators or primary criteria for selecting plant extracts for further investigation on SARS-CoV-2 cellular entry. According to the correlation analysis, the relationship between the binding inhibitory activity and the total phytochemical contents (i.e., total flavonoid and phenolic contents) was generally weak. This means

that the binding inhibitory activity of Zingiberaceae extracts is more likely related to specific phytochemicals (active compounds) than total phenolic or flavonoid contents. Thus, subsequent experiments on the inhibitory activity of selected plant extracts (*B. rotunda* extracts) were performed using the fractionated compounds. Furthermore, this knowledge suggests that total phenolic or flavonoid contents might not be an appropriate criterion for selecting plant extracts for further investigation of SARS-CoV-2 cellular entry.

All three *B. rotunda* rhizome extracts from different sources showed significant binding inhibitory activity against wild-type SARS-CoV-2 spike RBD–ACE2 binding. They contained six major compounds, with differences in their composition. The variation in phytochemical composition is likely due to the influence of plant cultivar and environmental factors [31]. Previous studies identified six major compounds in *B. rotunda*, namely narigenin 5-methyl ether, alpinetin, pinocembrin, pinostrobin, 4-hydroxypanduratin A, and panduratin A [25,27]. In an attempt to identify the fractionated compounds, the present study compared chromatograms and UV profiles to previously reported data. However, these are deemed insufficient for the identification of unknown compounds, as UV-spectra produce bands that are often very similar in closely related compounds. Thus, further experiments are required to identify these fractionated compounds from *B. rotunda* rhizome extracts using a structural identification technique like NMR spectroscopy, together with MS/MS data. Although the three *B. rotunda* rhizome extracts showed a difference in phytochemical profiles, they exhibited nonsignificant binding inhibitory activity. This might be because the most active compounds, Fraction V (tentatively identified as 4-hydroxypanduratin A) and Fraction VI (identified as panduratin A), are quite similar in all three extracts (Table 1). The inhibitory dose-response curve for binding inhibition against wild-type SAR-CoV-2–ACE2 was only carried out for panduratin A, yielding an IC_{50} of 0.38 $\mu\text{g/mL}$ (0.9 μM). A previous study also showed the potential of panduratin A as an inhibitor of SARS-CoV-2 cellular entry in Vero E6 cells, with an IC_{50} of 2.15 $\mu\text{g/mL}$ (5.30 μM) [22]. This is in line with the previously reported IC_{50} value (0.8–1.6 μM) on antiviral activity of panduratin A against wild-type SARS-CoV-2 using human induced pluripotent stem cell–derived cardiomyocytes [32]. According to the molecular docking study, panduratin A showed strong binding affinity for 2'-O-methyltransferase, main protease, RNA-dependent RNA polymerase, and the RBD of spike protein, an important protein in SARS-CoV-2 [33,34]. Besides panduratin A, 4-hydroxypanduratin A (Fraction V) is also an interesting compound, showing promising inhibitor activity against SARS-CoV-2 cellular entry, as observed in the present study. Vergoten and Bailly [34] also found that 4-hydroxypanduratin A had a higher binding affinity to SARS-CoV-2 main protease than panduratin A, likely due to the presence of a hydroxyl group. Both panduratin A and 4-hydroxypanduratin A are promising anti-inflammatory compounds that exhibit potent inhibitory activity against the production of proinflammatory cytokines, such as nitric oxide, prostaglandin E2, and tumor necrosis factor- α , by suppressing key enzymes involved in an inflammatory pathway [35–39]. Upon cellular entry, SARS-CoV-2 replicates and infects the host's cells, causing an excessive increase in proinflammatory cytokines and free radicals, which lead to tissue damage and hyperinflammation, the onset of multi-organ failure in COVID-19 patients [3]. For the treatment of COVID-19, compounds with multi-biological activities, importantly antioxidant, anti-inflammation, and antiviral, would thus be required. A recent in vivo study showed that although *A. paniculata* and *B. rotunda* extracts could not directly reduce viral load in the lungs, they could reduce inflammatory cytokines in Golden Syrian hamsters inoculated with the SARS-CoV-2 Delta variant [40].

The present study has some limitations. Although the findings reveal the potential of some Zingiberaceae plants against SARS-CoV-2 cellular entry, the sole use of the wild-type SARS-CoV-2 spike RBD protein limited the clinical relevance to current SARS-CoV-2 variants. Since the outbreak

in 2019, several SARS-CoV-2 variants have been identified worldwide, with mutations occurring particularly in the spike protein, influencing viral transmissibility, infectivity, and immune escape potential [12,41]. There is a possibility that phytochemicals exert similar binding inhibitory activity across various variants, as several phytochemicals, such as ursolic acid, betulinic acid, β -sitosterol, dioscin, quercetin, rutin, luteolin, and curcumin, displayed similar binding affinities to RBD spike proteins from five wild-type and four variants (Alpha, Beta, Delta, and Omicron) based on a molecular study [12]. The current findings need further validation in other tested systems, such as cell-based viral infection models, since the present study used only a biochemical assay. Another limitation is a lack of clarification of the underlying mechanism of SARS-CoV-2 cellular entry. The binding inhibitory activity of Zingiberaceae plants might be due to phytochemical binding to the spike protein and/or to ACE2. Previous studies have reported dual binding affinity of clove extracts and some phytochemicals for both the spike protein and ACE2, while others have reported high binding affinity only to the spike protein [30,42–44]. Although both pathways hamper viral cellular entry, the clinical application of ACE2 inhibitors needs to be carefully considered, as ACE2 plays an important role in the regulation of blood pressure. Therefore, future studies should validate the identified active compounds against currently circulating SARS-CoV-2 variants using variant-specific spike proteins and cell-based infection models to confirm their broader antiviral potential.

5. Conclusions

Overall, several Zingiberaceae extracts, particularly *A. biflorum*, *B. rotunda*, *C. aromatica*, *C. mangga*, *C. zedoaria*, *K. parviflora*, *Kaempferia* sp., and *Z. montanum*, exhibited inhibitory activity against the interaction between the wild-type SARS-CoV-2 spike protein and ACE2 in a biochemical binding assay. The observed binding inhibitory activity was not directly correlated with total phenolic or flavonoid contents, suggesting that specific phytochemicals rather than overall phytochemical abundance are responsible for the activity. Among the tested extracts, *B. rotunda* was identified as the most promising species, with panduratin A and 4-hydroxypanduratin A identified as the major binding inhibitory constituents. Panduratin A exhibited an IC_{50} of 0.38 μ g/mL. These findings highlight the potential of Zingiberaceae phytochemicals as candidates for further investigation targeting SARS-CoV-2 viral entry. However, as the present study was limited to a biochemical assay using the wild-type spike protein, further validation using clinically relevant SARS-CoV-2 variants, cell-based viral infection models, and in vivo studies is required before antiviral efficacy can be concluded.

Author contributions

Rungnapa Boonpawa: Conceptualization, methodology, investigation, visualization, writing—original draft preparation, and writing—reviewing and editing. Surassawadee Promyou: Conceptualization, supervision, and writing—reviewing and editing. All authors have read and approved the final version of the manuscript for publication.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Acknowledgments

This study was financially supported by Kasetsart University Research and Development Institute (KURDI), Project No. R-M 52.64.

Conflict of interest

The authors declare no conflict of interest.

References

1. Han DP, Penn-Nicholson A, Cho MW (2006) Identification of critical determinants on ACE2 for SARS-CoV entry and development of a potent entry inhibitor. *Virology* 350: 15–25. <https://doi.org/10.1016/j.virol.2006.01.029>
2. Lan J, Ge J, Yu J, et al. (2020) Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* 581: 215–220. <https://doi.org/10.1038/s41586-020-2180-5>
3. Montazersaheb S, Khatibi SMH, Hejazi MS, et al. (2022) COVID-19 infection: An overview on cytokine storm and related interventions. *Virol J* 19: 92. <https://doi.org/10.1186/s12985-022-01814-1>
4. Yang J, Petitjean SJL, Koehler M, et al. (2020) Molecular interaction and inhibition of SARS-CoV-2 binding to the ACE2 receptor. *Nat Commun* 11: 4541. <https://doi.org/10.1038/s41467-020-18319-6>
5. Khanna K, Raymond W, Jin J, et al. (2021) Thiol drugs decrease SARS-CoV-2 lung injury in vivo and disrupt SARS-CoV-2 spike complex binding to ACE2 in vitro. *BioRxiv*. https://doi.org/10.1164/ajrccm-conference.2022.205.1_MeetingAbstracts.A3546
6. Murae M, Shimizu Y, Yamamoto Y, et al. (2022) The function of SARS-CoV-2 spike protein is impaired by disulfide-bond disruption with mutation at cysteine-488 and by thiol-reactive N-acetyl-cysteine and glutathione. *Biochem Bioph Res Co* 597: 30–36. <https://doi.org/10.1016/j.bbrc.2022.01.106>
7. Aboul-Fotouh S, Mahmoud AN, Elnahas EM, et al. (2023) What are the current anti-COVID-19 drugs? From traditional to smart molecular mechanisms. *Virol J* 20: 241. <https://doi.org/10.1186/s12985-023-02210-z>
8. Liana D, Phanumartwiwath A (2022) Leveraging knowledge of Asian herbal medicine and its active compounds as COVID-19 treatment and prevention. *J Nat Med* 76: 20–37. <https://doi.org/10.1007/s11418-021-01575-1>
9. de Araújo FF, de Paulo Farias D, Neri-Numa IA, et al. (2021) Polyphenols and their applications: An approach in food chemistry and innovation potential. *Food Chem* 338: 127535. <https://doi.org/10.1016/j.foodchem.2020.127535>
10. Paraiso IL, Revel JS, Stevens JF (2020) Potential use of polyphenols in the battle against COVID-19. *Curr Opin Food Sci* 32: 149–155. <https://doi.org/10.1016/j.cofs.2020.08.004>
11. Badraoui R, Saoudi M, Hamadou WS, et al. (2022) Antiviral effects of artemisinin and its derivatives against SARS-CoV-2 main protease: computational evidences and interactions with ACE2 allelic variants. *Pharmaceuticals* 15. <https://doi.org/10.3390/ph15020129>
12. Sevimoglu T, Kızılpınar E, Çalışkaner ZO, et al. (2025) Uncovering potent natural phytochemicals targeting SARS-COV-2 spike protein variants: molecular dynamics insights. *Sci Rep* 15: 37927. <https://doi.org/10.1038/s41598-025-25242-7>

13. Wairata J, Fadlan A, Purnomo AS, et al. (2021) Total phenolic and flavonoid contents, antioxidant, antidiabetic and antiplasmodial activities of *Garcinia forbesii* King: A correlation study. *Arab J Chem* 15: 103541. <https://doi.org/10.1016/j.arabjc.2021.103541>
14. Hafshejani SF, Lotfi S, Rezvannejad E, et al. (2023) Correlation between total phenolic and flavonoid contents and biological activities of 12 ethanolic extracts of Iranian propolis. *Food Sci Nutr* 11: 4308–4325. <https://doi.org/10.1002/fsn3.3356>
15. Ndwandwe SL, Thibane VS, Gololo SS (2026) Evaluation of antiviral activity indicators in selected medicinal plants used traditionally for treatment of respiratory viral infections. *BMC Complement Med* 26: 141. <https://doi.org/10.1186/s12906-026-05322-5>
16. Sharifi-Rad M, Varoni EM, Salehi B, et al. (2017) Plants of the genus *Zingiber* as a source of bioactive phytochemicals: from tradition to pharmacy. *Molecules* 22. <https://doi.org/10.3390/molecules22122145>
17. Mogha NG (2024) Ethnobotanical study of medicinal plants used in management of COVID-19 in Dar es Salaam and Morogoro Regions, Tanzania. *Ethnobotany Res Appl* 29: 1–16. <https://doi.org/10.32859/era.29.2.1-16>
18. Thangjam NM, Laldingliani TBC, Kumar A, et al. (2024) Ethnobotanical documentation of medicinal plants used during COVID-19 by the Meitei Community in Manipur, India. *Ethnobotany Res Appl* 29: 1–24. <https://doi.org/10.32859/era.29.62.1-24>
19. Burapan S, Kim M, Paisooksantivatana Y, et al. (2020) Thai Curcuma species: antioxidant and bioactive compounds. *Foods* 9. <https://doi.org/10.3390/foods9091219>
20. Morvaridzadeh M, Fazelian S, Agah S, et al. (2020) Effect of ginger (*Zingiber officinale*) on inflammatory markers: A systematic review and meta-analysis of randomized controlled trials. *Cytokine* 135: 155224. <https://doi.org/10.1016/j.cyto.2020.155224>
21. Verma AK, Kumar V, Singh S, et al. (2021) Repurposing potential of Ayurvedic medicinal plants derived active principles against SARS-CoV-2 associated target proteins revealed by molecular docking, molecular dynamics and MM-PBSA studies. *Biomed Pharmacother* 137: 111356. <https://doi.org/10.1016/j.biopha.2021.111356>
22. Kanjanasirirat P, Suksatu A, Manopwisedjaroen S, et al. (2020) High-content screening of Thai medicinal plants reveals *Boesenbergia rotunda* extract and its component Panduratin A as anti-SARS-CoV-2 agents. *Sci Rep* 10: 19963. <https://doi.org/10.1038/s41598-020-77003-3>
23. Attard E (2013) A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. *Cent Eur J Biol* 8: 48–53. <https://doi.org/10.2478/s11535-012-0107-3>
24. Herald TJ, Gadgil P, Tilley M (2012) High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. *J Sci Food Agr* 92: 2326–2331. <https://doi.org/10.1002/jsfa.5633>
25. Adhikari D, Gong DS, Oh SH, et al. (2020) Vasorelaxant effect of *Boesenbergia rotunda* and its active ingredients on an isolated coronary artery. *Plants* 9: 1688. <https://doi.org/10.3390/plants9121688>
26. Liu X, Raghuvanshi R, Ceylan FD, et al. (2020) Quercetin and its metabolites inhibit recombinant human angiotensin-converting enzyme 2 (ACE2) activity. *J Agr Food Chem* 68: 13982–13989. <https://doi.org/10.1021/acs.jafc.0c05064>
27. Tan BC, Tan SK, Wong SM, et al. (2015) Distribution of flavonoids and cyclohexenyl chalcone derivatives in conventional propagated and in vitro-derived field-grown *Boesenbergia rotunda* (L.) Mansf. *Evid-Based Compl Alt* 2015: 451870. <https://doi.org/10.1155/2015/451870>

28. Goc A, Sumera W, Rath M, et al. (2021) Phenolic compounds disrupt spike-mediated receptor-binding and entry of SARS-CoV-2 pseudo-virions. *PLoS One* 16: e0253489. <https://doi.org/10.1371/journal.pone.0253489>
29. Gopinath K, Jokinen EM, Kurkinen ST, et al. (2020) Screening of natural products targeting SARS-CoV-2–ACE2 receptor interface—a MixMD based HTVS pipeline. *Front Chem* 8. <https://doi.org/10.1371/journal.pone.0253489>
30. Li Y, Liu Z, Zeng M, et al. (2022) Chemical compositions of clove (*Syzygium aromaticum* (L.) Merr. & L.) extracts and their potentials in suppressing SARS-CoV-2 spike protein-ACE2 binding, inhibiting ACE2, and scavenging free radicals. *J Agr Food Chem* 70: 14403–14413. <https://doi.org/10.1021/acs.jafc.2c06300>
31. Thomya S, Wongkaew M, Bundithya W, et al. (2023) Relationship between phenotypes and chemical profiling of *Boesenbergia rotunda* collected from different habitats of Northern Thailand. *Agronomy* 13: 1435. <https://doi.org/10.3390/agronomy13061435>
32. Linn AK, Manopwisedjaroen S, Kanjanasirirat P, et al. (2024) Unveiling the antiviral properties of panduratin A through SARS-CoV-2 infection modeling in cardiomyocytes. *Int J Mol Sci* 25: 1427. <https://doi.org/10.3390/ijms25031427>
33. Boonserm P, Khunrae P, Sutthibutpong T (2023) A computational study on the molecular mechanisms of panduratin A as a potential inhibitor on SARS-CoV-2 protein targets. *Heliyon* 9: e12780. <https://doi.org/10.1016/j.heliyon.2022.e12780>
34. Vergoten G, Bailly C (2023) Interaction of panduratin A and derivatives with the SARS-CoV-2 main protease (m(pro)): A molecular docking study. *J Biomol Struct Dyn* 41: 6834–6844. <https://doi.org/10.1080/07391102.2022.2112618>
35. Tanigaki R, Takahashi R, Nguyen MTT, et al. (2019) 4-Hydroxypanduratin A and isopanduratin A inhibit tumor necrosis factor α -stimulated gene expression and the nuclear factor κ B-dependent signaling pathway in human lung adenocarcinoma A549 cells. *Biol Pharm Bull* 42: 26–33. <https://doi.org/10.1248/bpb.b18-00457>
36. Tewtrakul S, Subhadhirasakul S, Karalai C, et al. (2009) Anti-inflammatory effects of compounds from *Kaempferia parviflora* and *Boesenbergia pandurata*. *Food Chem* 115: 534–538. <https://doi.org/10.1016/j.foodchem.2008.12.057>
37. Tuchinda P, Reutrakul V, Claeson P, et al. (2002) Anti-inflammatory cyclohexenyl chalcone derivatives in *Boesenbergia pandurata*. *Phytochemistry* 59: 169–173. [https://doi.org/10.1016/S0031-9422\(01\)00451-4](https://doi.org/10.1016/S0031-9422(01)00451-4)
38. Yun JM, Kwon H, Hwang JK (2003) In vitro anti-inflammatory activity of panduratin A isolated from *Kaempferia pandurata* in RAW264.7 cells. *Planta Med* 69: 1102–1108. <https://doi.org/10.1055/s-2003-45190>
39. Zhang L, Jiang Q, Wang X, et al. (2023) *Boesenbergia rotunda* displayed anti-inflammatory, antioxidant and anti-apoptotic efficacy in doxorubicin-induced cardiotoxicity in rats. *Sci Rep* 13: 11398. <https://doi.org/10.1038/s41598-023-38560-5>
40. Kongsomros S, Boonyarattanasoonthorn T, Phongphaew W, et al. (2024) In vivo evaluation of *Andrographis paniculata* and *Boesenbergia rotunda* extract activity against SARS-CoV-2 Delta variant in Golden Syrian hamsters: Potential herbal alternative for COVID-19 treatment. *J Tradit Compl Med* 14: 598–610. <https://doi.org/10.1016/j.jtcme.2024.05.004>
41. Verma S, Patil VM, Gupta MK (2022) Mutation informatics: SARS-CoV-2 receptor-binding domain of the spike protein. *Drug Discov Today* 27: 103312. <https://doi.org/10.1016/j.drudis.2022.06.012>

42. Carino A, Moraca F, Fiorillo B, et al. (2020) Hijacking SARS-CoV-2/ACE2 receptor interaction by natural and semi-synthetic steroidal agents acting on functional pockets on the receptor binding domain. *Front Chem* 8. <https://doi.org/10.3389/fchem.2020.572885>
43. Singh P, Chauhan SS, Pandit S, et al. (2022) The dual role of phytochemicals on SARS-CoV-2 inhibition by targeting host and viral proteins. *J Tradit Compl Med* 12: 90–99. <https://doi.org/10.1016/j.jtcme.2021.09.001>
44. Muchtaridi M, Fauzi M, Ikram NKK, et al. (2020) Natural flavonoids as potential angiotensin-converting enzyme 2 inhibitors for anti-SARS-CoV-2. *Molecules* 25: 3980. <https://doi.org/10.3390/molecules25173980>



AIMS Press

© 2026 the Author(s), licensee AIMS Press. This is an open access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0>)