

Hydroalcoholic extract of *Salvia officinalis*: Antioxidant properties and *in vitro* screening against SARS-CoV-2 Spike–ACE2 interaction

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SUMMARY

Introduction: *Salvia officinalis* L. (sage) belongs to the Lamiaceae family and is native to the Middle East and Mediterranean regions. Sage extract is an excellent source of flavonoids and polyphenolic compounds, such as rosmarinic acid. Rosmarinic acid, a phenolic compound found in various Lamiaceae plants, exhibits antioxidant, anti-inflammatory, antibacterial, and antiviral activities. **Objective:** This study aimed to characterize the antioxidant capacity of the hydroalcoholic extract (SO-CWB) of the *S. officinalis* L. and to evaluate its ability to inhibit the interaction between ACE2 and Spike proteins in an *in vitro* assay. **Methodology:** The extract was characterized through analyses of total phenolic compounds, flavonoids, reducing sugars, antioxidant activity (DPPH, ABTS, and FRAP), and evaluated through a screening assay for inhibitors of the interaction between the Spike protein of the receptor-binding domain (RBD) of the wild-type (WT) variant of SARS-CoV-2. **Results:** The hydroethanolic extract showed concentrations of rosmarinic acid (5.1 ± 0.8) $\mu\text{g mg}^{-1}$, total phenolic compounds (60.9 ± 0.3) $\mu\text{g}_{\text{GAE}} \text{mg}^{-1}$, flavonoid compounds (30.11 ± 0.4) $\mu\text{g}_{\text{QE}} \text{mg}^{-1}$, and reduced sugar (352.2 ± 31.8) $\mu\text{g}_{\text{GE}} \text{mg}^{-1}$. Additionally, it exhibited antioxidant activity against DPPH (135.5 ± 3.8) $\mu\text{mol}_{\text{TE}} \text{L}^{-1} \text{mg}^{-1}$, ABTS (221.2 ± 2.7) $\mu\text{mol}_{\text{TE}} \text{L}^{-1} \text{mg}^{-1}$, and FRAP (326.1 ± 20.9) $\mu\text{mol}_{\text{FeSO}_4} \text{L}^{-1} \text{mg}^{-1}$. The extract and the rosmarinic acid standard were evaluated for their ability to inhibit the interaction between the RBD Spike protein of the wild-type (WT) variant of SARS-CoV-2. The extract and rosmarinic acid did not influence the interaction between ACE2 and Spike proteins in the assay (activity >90%). So, the standard hesperidin was tested as a comparative and to evaluate the efficacy of the test, and hesperidin showed binding-inhibition activity against Spike-ACE2. **Conclusion:** Although the extract and rosmarinic acid did not interfere with

Spike–ACE2 binding, strong antioxidant activity, suggesting potential relevance in mitigating oxidative stress and reactive oxygen species (ROS) during COVID-19.

Keywords: *Salvia officinalis* L.; rosmarinic acid; biological activity; Spike protein; SARS-CoV-2.

RESUMÉN

Extracto hidroalcohólico de *Salvia officinalis*: Propiedades antioxidantes y detección *in vitro* de la interacción entre la proteína Spike y la ECA2 del SARS-CoV-2

Introducción: *Salvia officinalis* L. (salvia) pertenece a la familia Lamiaceae y es originaria del Oriente Medio y de las regiones mediterráneas. El extracto de salvia es una excelente fuente de flavonoides y de compuestos polifenólicos, como el ácido rosmarínico. El ácido rosmarínico, un compuesto fenólico presente en diversas plantas de la familia Lamiaceae, exhibe actividades antioxidantes, antiinflamatorias, antibacterianas y antivirales. **Objetivo:** Caracterizar la capacidad antioxidante de un extracto hidroalcohólico (SO-CWB) de *Salvia officinalis* L. y evaluar su capacidad para inhibir la interacción entre las proteínas ACE2 y Spike en un ensayo *in vitro*. **Metodología:** El extracto fue caracterizado mediante análisis de compuestos fenólicos totales, flavonoides y azúcares reductores, y de la actividad antioxidante (DPPH, ABTS y FRAP), y evaluado en un ensayo de cribado para inhibidores de la interacción entre la proteína Spike del dominio de unión al receptor (RBD) de la variante silvestre (WT) del SARS-CoV-2. **Resultados:** El extracto hidroetanólico mostró concentraciones de ácido rosmarínico ($5.06 \pm 0.8 \mu\text{g mg}^{-1}$), compuestos fenólicos totales ($60.9 \pm 0.3 \mu\text{g GAE mg}^{-1}$), compuestos flavonoides ($30.11 \pm 0.4 \mu\text{g QE mg}^{-1}$) y azúcares reductores ($352.2 \pm 31.8 \mu\text{g GE mg}^{-1}$). Además, exhibió actividad antioxidante frente a DPPH ($135.5 \pm 3.8 \mu\text{mol TE L}^{-1} \text{mg}^{-1}$), ABTS ($221.2 \pm 2.7 \mu\text{mol TE L}^{-1} \text{mg}^{-1}$) y FRAP ($326.1 \pm 20.9 \mu\text{mol FeSO}_4 \text{ L}^{-1} \text{mg}^{-1}$). El extracto y el estándar de ácido rosmarínico se evaluaron mediante un ensayo de tamizaje para identificar inhibidores de la interacción entre el dominio de unión al receptor (RBD) de la proteína Spike de la variante de tipo salvaje (WT) del SARS-CoV-2. El extracto y el ácido rosmarínico no influyeron en la interacción entre las proteínas ACE2 y Spike en el ensayo (actividad > 90%). Por ello, se probó la hesperidina estándar como comparación, y la evaluación de la eficacia, tanto del extracto como de la hesperidina, mostró actividad en la inhibición de la unión entre Spike y ACE2. **Conclusión:** Aunque el extracto y el ácido rosmarínico no interfirieron en la unión Spike–ACE2, debido a su fuerte actividad antioxidante, el extracto puede considerarse relevante para contrarrestar la sobreproducción de especies reactivas de oxígeno (ROS) y el estrés oxidativo durante la COVID-19.

Palabras clave: *Salvia officinalis* L.; ácido rosmarínico; actividad biológica; proteína Spike; SARS-CoV-2.

RESUMO

Extrato hidroalcoólico de *Salvia officinalis*: propriedades antioxidantes e triagem *in vitro* contra a interação Spike-ACE2 do SARS-CoV-2

Salvia officinalis L. (sálvia) pertence à família Lamiaceae e é nativa do Oriente Médio e das regiões mediterrâneas. O extrato de sálvia é uma excelente fonte de flavonoides e de compostos polifenólicos, como o ácido rosmarínico. O ácido rosmarínico, um composto fenólico presente em diversas plantas da família Lamiaceae, apresenta atividades antioxidantes, anti-inflamatórias, antibacterianas e antivirais. **Objetivo:** Este estudo teve como objetivo caracterizar a capacidade antioxidante de um extrato hidroalcoólico (SO-CWB) de *Salvia officinalis* L. e avaliar sua capacidade de inibir a interação entre as proteínas ACE2 e Spike em um ensaio *in vitro*. **Metodologia:** O extrato foi caracterizado por meio de análises de compostos fenólicos totais, flavonoides, açúcares reductores e atividade antioxidante (DPPH, ABTS e FRAP), e avaliado em um ensaio de triagem para inibidores da interação entre a proteína Spike do domínio de ligação ao receptor (RBD) da variante selvagem (WT) do SARS-CoV-2. **Resultados:** O extrato hidroetanólico apresentou concentrações de ácido rosmarínico ($5,06 \pm 0,8 \mu\text{g mg}^{-1}$), compostos fenólicos totais ($60,9 \pm 0,3 \mu\text{g}_{\text{GAE}} \text{mg}^{-1}$), compostos flavonoides ($30,11 \pm 0,4 \mu\text{g}_{\text{QE}} \text{mg}^{-1}$) e açúcares reductores ($352,2$

$\pm 31,8 \mu\text{g}_{\text{GE}} \text{mg}^{-1}$). Além disso, apresentou atividade antioxidante contra DPPH ($135,5 \pm 3,8 \mu\text{mol}_{\text{TE}} \text{L}^{-1} \text{mg}^{-1}$), ABTS ($221,2 \pm 2,75 \mu\text{mol}_{\text{TE}} \text{L}^{-1} \text{mg}^{-1}$) e FRAP ($326,1 \pm 20,9 \mu\text{mol}_{\text{FeSO}_4} \text{L}^{-1} \text{mg}^{-1}$). O extrato e o padrão de ácido rosmarínico foram avaliados por meio de um ensaio de triagem para inibidores da interação entre o domínio de ligação ao receptor (RBD) da proteína Spike da variante do tipo selvagem (WT) do SARS-CoV-2. O extrato e o ácido rosmarínico não influenciaram a interação entre as proteínas ACE2 e Spike no ensaio (atividade > 90%). Assim, o padrão hesperidina foi utilizado como comparativo, e a avaliação da eficácia do extrato e da hesperidina demonstrou atividade na inibição da ligação entre Spike e ACE2. **Conclusão:** Embora o extrato e o ácido rosmarínico não tenham interferido na ligação Spike-ACE2, devido à sua forte atividade antioxidante, o extrato pode ser considerado relevante para contrabalançar a superprodução de espécies reativas de oxigênio (ROS) e o estresse oxidativo durante a COVID-19.

Palavras-chave: *Salvia officinalis* L.; ácido rosmarínico; atividade biológica; proteína Spike; SARS-CoV-2.

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric Reducing Antioxidant Power; GAE, Gallic acid equivalent; QE, Quercetin equivalent. TE, Trolox equivalent, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; GE, Glucose equivalent.

1. INTRODUCTION

Salvia officinalis L., commonly known as sage, is a member of the Lamiaceae family and is native to the Middle East and Mediterranean regions [1-3]. It is rich in phytochemicals, such as flavonoids, polyphenolic compounds like rosmarinic acid, alkaloids, carbohydrates, terpenes, and terpenoids, monoterpenoids, diterpenoids, triterpenoids, and sesquiterpenoids [1-3]. Sage has various traditional uses due to its antioxidant, anti-inflammatory, antibacterial, and antiviral properties, which induce pain relief, reduce inflammation, and help with ulcers and diuretic effects [1-3]. These effects are partly due to the presence of rosmarinic acid, a major phenolic compound in sage, which has strong antioxidant and anti-inflammatory activities [3]. Studies suggest potential in treating a lot of types of diseases, like Alzheimer's disease, diabetes Mellitus, depression, heart diseases, and even cancer [1-4]. Other studies have indicated the use of sage extract for memory, concentration, and cognitive function.

According to molecular docking studies, rosmarinic acid shows potential against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) by inhibiting viral entry into cells through interactions with the main protease (Mpro) and the spike protein [5]. Additionally, due to its antioxidant properties, rosmarinic acid and sage could be effective against the overproduction of Reactive Oxygen Species (ROS), which are produced by neutrophils and macrophages during severe infections, like COVID-19, and could express the nuclear factor erythroid 2-related factor 2 (Nrf2) and small musculoaponeurotic fibrosarcoma proteins (sMAF) interfering with the production of ROS [6].

In this context, this study aimed to characterize the antioxidant capacity of a hydroalcoholic extract (SO) from *Salvia officinalis* L. and to evaluate its ability to inhibit the interaction between ACE2 and Spike proteins in an *in vitro* assay, in search of new alternative therapeutic options to treat the symptoms and complications caused by SARS-CoV-2. This virus causes Coronavirus Disease 2019 (COVID-19).

2. METHODOLOGY

2.1. Reagents

The solvents used were ethanol (99.7%, Êxodo Científica Química Fina, Sumaré, Brazil), hydrochloric acid (Neon Comercial Ltda, São Paulo, Brazil), and Folin–Ciocalteu (Cromato Produtos Químicos, São Paulo, Brazil). The standards were: rosmarinic acid (Sigma-Aldrich, R4033), gallic acid (Sigma-Aldrich, G7384), Quercetin (Sigma-Aldrich, Q4951), Trolox (97%, Sigma-Aldrich, 238813), DPPH (Sigma-Aldrich, D9132), ABTS (Sigma-Aldrich, A1888), and glucose (Sigma-Aldrich, G7021). The reagents used were aluminum chloride (98%, Sigma-Aldrich, 206911), anhydrous sodium carbonate (Casa da Química, Diadema, Brazil), ferric chloride (Biotec Reagentes Analíticos, Pinhais, Brasil), potassium persulfate (Sigma-Aldrich, St. Louis, EUA), and ferrous sulfate (Cromato Produtos Químicos, São Paulo, Brazil). Other chemicals or standards were commercial products of the highest purity available.

2.2. Plant material and extraction

Sage leaves, cultivated in Curitiba, Brazil, by Juliana B. B. Maurer, were dried in an oven with forced-air circulation at 40 °C for 3 days, then ground into a fine powder. To prepare the extract, 12 g of dry sage powder was dissolved in 120 mL of 70% (v/v) ethanol, mixed, and stored in the dark for 7 days with daily agitation at room temperature (25 °C), according to guidelines described in the Brazilian Pharmacopoeia (2021) [7]. The resulting solution was filtered through Whatman No. 1 filter paper, then evaporated and lyophilized, and characterized by spectroscopic analysis. For all analyses, the extract was prepared in 70% (v/v) ethanol at a minimum concentration of 1 mg·mL⁻¹ and was called SO-CWB.

2.3. Spectrophotometric characterization

2.3.1. Total phenolic content - TPC

Total phenolics in the SO-CWB extract were quantified using the Folin–Ciocalteu method [8]. 20 µL of sample was mixed with 100 µL of 10% (v/v) Folin–Ciocalteu reagent and allowed to react at room temperature for 5 minutes. Subsequently, 75 µL of 7.5% (w/v) sodium carbonate was added, and the mixture was incubated in the dark for 40 minutes. Absorbance was recorded at 760 nm using a BioTek EPOCH microplate reader (Agilent, USA). Gallic acid served as the calibration standard, and results were expressed as µg gallic acid equivalent per mg of extract (µg_{GAE}·mg⁻¹).

2.3.2. Flavonoid content

Flavonoid content was assessed by reacting 100 µL of extract with an equal volume of 2% (w/v) aluminum chloride solution. After a 10-minute reaction at room temperature, the absorbance was measured at 425 nm. Quercetin was used as the standard, and results were expressed as µg quercetin equivalent per mg of extract (µg_{QE}·mg⁻¹) [9].

2.3.3. Reducing Sugar content by dinitrosalicylic acid – DNS

Sugar levels were reduced using the 3,5-dinitrosalicylic acid (DNS) method. Equal volumes (250 µL) of extract and DNS solution were combined and heated in a boiling water bath for 5 minutes. The reaction was then diluted with 2.5 mL of cold distilled water and read at 540 nm. Glucose was the reference standard, and results were reported as µg glucose equivalents per mg of extract (µg_{GE}·mg⁻¹) [10, 11].

2.4. Antioxidant activities

Given the complexity of plant extracts and the diversity of antioxidant mechanisms, multiple *in vitro* assays were used to evaluate the antioxidant potential of SO-CWB [12] comprehensively.

2.4.1. FRAP assay

Ferric reducing power was assessed using the FRAP method [13]. A total of 20 μL of extract, 30 μL of distilled water, and 200 μL of freshly prepared FRAP reagent (acetate buffer pH 3.6, TPTZ in HCl, and FeCl_3 in a 10:1:1 v/v ratio) were mixed and incubated at 37 °C for 8 minutes. Absorbance was measured at 595 nm. Results were expressed as FeSO_4 equivalents.

2.4.2. DPPH assay

The antioxidant capacity of SO-CWB was determined using the DPPH radical scavenging method [8]. The experiment was conducted in triplicate by mixing 66 μL of the sage extract with 134 μL of a DPPH solution in ethanol (150 $\mu\text{mol}\cdot\text{L}^{-1}$). After gentle agitation, the mixtures were incubated at room temperature in the dark for 30 minutes. Absorbance readings at 517 nm were obtained using a BioTek EPOCH UV-Vis microplate spectrophotometer (Agilent, USA). Antioxidant activity was expressed as Trolox equivalent.

2.4.3. ABTS assay

The antioxidant capacity of SO-CWB was determined using the $\text{ABTS}^{\bullet+}$ radical method [8]. The radical was generated by reacting with potassium persulfate and adjusted to an absorbance of 0.700 ± 0.002 . For the assay, 20 μL of sample was added to 220 μL of $\text{ABTS}^{\bullet+}$ solution and incubated for 6 minutes in the dark. Absorbance was then measured at 734 nm. Antioxidant activity was expressed in Trolox equivalents.

2.4.4. Quantification of Rosmarinic Acid by HPLC

To quantify rosmarinic acid, the methodology adapted from Soldera-Silva *et al.* (2018) [14] was used. Rosmarinic acid was identified and quantified by high-performance liquid chromatography (HPLC) using an Agilent 1200 chromatograph (Santa Clara, California, USA), equipped with a vacuum degasser (G1322A), a quaternary pump (G1311A), a thermostatted column compartment (G1316A), and a multiple wavelength (MWD) UV-Vis detector (G1365D). The instrument was operated at a wavelength of 330 nm. Chromatographic separation was performed on an Agilent C-18 column (150 mm $\times$ 4.6 mm, 5 μm particles) with a single injection of 20 μL . The mobile phase consisted of formic acid (1% v/v) in ultrapure water (A) and HPLC-grade acetonitrile (B). The gradient applied was 0-5 min, 5% B; 5-10 min, 10% B; 10-20 min, 30% B; 20-30 min, 50% B with a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$ at 25 °C. A stock solution of the rosmarinic acid standard was prepared in HPLC-grade methanol at 1mg/mL and filtered through a hydrophilic cellulose acetate filter (0.22 μm pore size, non-sterile). Six different concentrations (200 to 3.125 $\mu\text{g}\cdot\text{mL}^{-1}$) were injected (20 μL) into the HPLC system in triplicate, and the analytical curve obtained by plotting peak area versus concentration showed a correlation coefficient of $R^2 = 0.999$ ($y = 135420.86 + 65162.39x$), indicating linearity. The limit of detection (LOD) was 11.78 $\mu\text{g}\cdot\text{mL}^{-1}$ (95% confidence), and the limit of quantification (LOQ) was 17.33 $\mu\text{g}\cdot\text{mL}^{-1}$ (95% confidence). The sage extract concentration used for quantification was 5 $\text{mg}\cdot\text{mL}^{-1}$, and the experiment was performed in triplicate.

2.4.5. Screening of SARS-CoV-2 Spike Protein Inhibitor

The inhibition of Spike-ACE2 interaction was measured employing the SARS-CoV-2 Inhibitor Screening Assay kit (EP-107, ACROBiosystems, Beijing, China) for the wild-type variant, with the ELISA (Enzyme Linked Immuno Sorbent Assay) reading platform. All reagents were used

from the same kit in individual experiments, and the assays were performed according to the manufacturer's protocol. SO-CWB and standard stock solutions were prepared in 70% (v/v) ethanol. The extract was diluted in dilution buffer to concentrations ranging from 1 to 4 mg·mL⁻¹, while the standards (rosmarinic acid and hesperidin) were diluted to concentrations from 0.1 to 0.4 mg·mL⁻¹. Rosmarinic acid and hesperidin were tested at concentrations of 277-1108 µM and 164-491 µM, respectively.

Samples (extract, standard as rosmarinic acid and hesperidin, and inhibitor, 50 µL) were added to the wells of the plate with pre-coated human ACE2 (Angiotensin Converting Enzyme 2), followed by the addition of 50 µL of HRP-SARS-CoV-2 Spike RBD, sealed, and incubated for 1 h at 37 °C in the dark. Afterward, the supernatant was removed, and the cells were washed with wash buffer (300 µL × 3 times). Then, the plate was dried, and 100 µL of the substrate solution was added. This plate was sealed and incubated for 20 min at 37 °C in the dark. The reaction was stopped by adding 50 µL of a stop solution. Absorbance was measured at 450 nm using a microplate reader UV-visible spectrophotometer (BioTek Instruments, Winooski, USA).

This assay was also tested by preincubating the samples with the WT variant. It was tested by preincubating 70 µL of the extract and standard with 70 µL of the Spike protein in a 96-well plate (V-bottom) for 30 min at 25 °C in the dark, then adding 100 µL of the mix (sample-test+Spike) to the pre-coated human ACE2. It was sealed with adhesive paper and incubated for 1 h at 37 °C in the dark. Afterward, the liquid was removed, and the wells were washed with wash buffer (300 µL × 3 times) using a washing plate (Agilent, USA). After this, the plate was dried, and 100 µL of the substrate solution was added. This plate was sealed and incubated for 20 min at 37 °C in the dark. The reaction was stopped by adding 50 µL of a stop solution. Absorbance was measured at 450 nm using a microplate reader UV-vis spectrophotometer (BioTek Instruments, Winooski, USA).

The activity of the test samples relative to the binding of ACE2 to HRP-SARS-CoV-2 Spike RBD proteins was compared with the results for the Spike protein inhibitor and the positive control (100% binding). So, the higher the % value of the activity, the greater the interaction between the ACE2 protein and the Spike protein.

To calculate the binding activity between ACE2 (Angiotensin-Converting Enzyme 2) and the Spike protein (HRP-SARS-CoV-2 Spike RBD), the absorbance values measured at 450 nm in Equation (1) below are applied.

$$\text{Activity \%} = \frac{\text{Sample Absorbance}}{\text{Positive control Absorbance}} \times 100\% \quad (1)$$

All treatments were done at least in triplicate, and two independent experiments were performed.

3. RESULTS AND DISCUSSION

3.1. Characterization by spectrophotometric and chromatographic methods

Table 1 shows the colorimetric characterization results obtained with the hydroalcoholic sage leaves extract (SO-CWB).

Table 1. Colorimetric characterization results and antioxidant activity of SO-CWB (hydroalcoholic sage extract).

Parameter	Mean $\pm$ SD
Total phenolic ($\mu\text{g}_{\text{GAE}}\cdot\text{mg}^{-1}$)	60.9 $\pm$ 0.3
Flavonoid ($\mu\text{g}_{\text{QE}}\cdot\text{mg}^{-1}$)	30.11 $\pm$ 0.4
Rosmarinic acid ($\mu\text{g}\cdot\text{mg}^{-1}$)	5.06 $\pm$ 0.8
DNS ($\mu\text{g}_{\text{GE}}\cdot\text{mg}^{-1}$)	352.2 $\pm$ 31.8
FRAP ($\mu\text{mol}_{\text{FeSO}_4\cdot\text{L}^{-1}}\cdot\text{mg}^{-1}$)	326.1 $\pm$ 20.9
DPPH ($\mu\text{mol}_{\text{TE}}\cdot\text{L}^{-1}\cdot\text{mg}^{-1}$)	135.5 $\pm$ 3.8
ABTS ($\mu\text{mol}_{\text{TE}}\cdot\text{L}^{-1}\cdot\text{mg}^{-1}$)	221.2 $\pm$ 2.75

DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP, Ferric Reduction Antioxidant Power; GAE, Gallic acid equivalent; QE, Quercetin equivalent; DNS, 3,5-dinitrosalicylic acid; TE, Trolox equivalent. SD, standard deviation. All analyses were performed in triplicate n=3).

The extract, with a yield of 11% (w/w), showed a total phenolic concentration of 60.9 $\mu\text{g}_{\text{GAE}}\cdot\text{mg}^{-1}$ of SO-CWB. Previous studies have demonstrated significant variations of these compounds in different extracts of *Salvia officinalis*. For example, a total phenolic content of 31.12 $\mu\text{g}_{\text{GAE}}\cdot\text{mg}^{-1}$ was reported in a sage hydroalcoholic extract (20% w/v). In comparison, concentrations between 70.5 and 176.6 $\mu\text{g}_{\text{GAE}}\cdot\text{mg}^{-1}$ were obtained in hydromethanolic extracts of sage leaves from distinct regions of Morocco [15, 16]. It was observed that total phenolic contents ranged from 0.339 to 2.337 $\mu\text{g}_{\text{GAE}}\cdot\text{mg}^{-1}$ of extract when evaluating the effect of different drying methods of the plant material [17].

For flavonoid content, a value of 30.11 $\mu\text{g}_{\text{QE}}\cdot\text{mg}^{-1}$ was obtained, which is higher than those found by Khyia *et al.* (2021), who reported concentrations between 1.92 and 9.11 $\mu\text{g}_{\text{QE}}\cdot\text{mg}^{-1}$ of extract [16]. Regarding rosmarinic acid, an essential phenolic acid with antioxidant and anti-inflammatory properties, the concentration was 5.06 $\pm$ 0.8 $\mu\text{g}\cdot\text{mg}^{-1}$. Hamrouni-Sellami *et al.* (2013) reported quantities ranging from 12.27 to 176.09 $\mu\text{g}\cdot\text{mg}^{-1}$ of extract across different extractions [17].

The extract's antioxidant activity was high across the three techniques evaluated, with concentrations of 326.1 $\mu\text{mol}_{\text{FeSO}_4\cdot\text{L}^{-1}}\cdot\text{mg}^{-1}$ for FRAP, 135.5 $\mu\text{mol}_{\text{TE}}\cdot\text{L}^{-1}\cdot\text{mg}^{-1}$ for DPPH, and 221.2 $\mu\text{mol}_{\text{TE}}\cdot\text{L}^{-1}\cdot\text{mg}^{-1}$ for ABTS. Mokhtari *et al.* (2023) obtained 69,21 $\text{mmol}_{\text{FeSO}_4\cdot\text{L}^{-1}}\cdot\text{mg}^{-1}$ using the FRAP method [18]. Antioxidant activity correlates with the high content of phenolic compounds and flavonoids in the extract [19].

Antioxidant compounds can neutralize ROS, reduce oxidative stress, and modulate the inflammatory response. *In vivo* studies have shown that *Salvia officinalis* extract has antioxidant and anti-inflammatory properties [3]. These effects are partly related to the presence of rosmarinic acid, one of the main phenolic compounds present in sage [3]. For example, the extract increased the resistance of rat hepatocytes to oxidative stress. It protected these cells from DNA damage caused by di-methoxy naphthoquinone and hydrogen peroxide by increasing glutathione peroxidase activity [20].

Rosmarinic acid has well-known antioxidant and anti-inflammatory properties [2, 21]. The reduction of reactive oxygen species evidences its antioxidant activity, increases in antioxidant enzyme activities (SOD, CAT, and GSH-Px), modulation of NADPH oxidases, and decreased lipid peroxidation [22]. These effects are mainly associated with the activation of the Nrf2 pathway, which improves resistance to oxidative stress and helps maintain cellular

redox balance [23]. The anti-inflammatory properties of rosmarinic acid include the downregulation of COX-2 expression and the inhibition of pro-inflammatory cytokine production [2, 3, 21, 24].

The antioxidant capacity is relevant in the context of COVID-19. Infection with SARS-CoV-2 can lead to excessive production of reactive oxygen species (ROS), contributing to oxidative stress, activation of the Nrf2 pathway, and tissue damage [25]. The excessive production of reactive oxygen species (ROS) during SARS-CoV-2 infection represents a potential target for therapeutic intervention to support the system affected by the disease [6]. Additionally, SARS-CoV-2 infection is associated with an exacerbated inflammatory response known as cytokine storm, characterized by the massive release of pro-inflammatory mediators such as interleukins (IL-6, IL-1 β) and tumor necrosis factor-alpha (TNF- α) [26].

Thus, the antioxidant and anti-inflammatory properties of rosmarinic acid support its potential use as an adjuvant in diseases with excessive inflammation, including SARS-CoV-2 infection. In severe COVID-19, neutrophils and macrophages produce high levels of reactive oxygen species (ROS), which contribute to tissue damage and disease progression [3, 24]. Rosmarinic acid is a strong antioxidant that acts partly by activating the Nrf2 pathway [25]. This activation increases the production of antioxidant enzymes, helping reduce oxidative stress, decrease COVID-19-related inflammation, and restore redox balance in cells. It may also support the repair of tissues damaged by viral infection and excess ROS, especially in organs such as the lungs and the heart [3, 6, 27–31].

Considering its chemical composition and antioxidant capacity, the SO-CWB extract may have therapeutic potential in inflammatory conditions such as COVID-19. Therefore, it can be evaluated in cellular models related to oxidative stress and hyperinflammatory signaling pathways involved in SARS-CoV-2 infection.

3.2. Screening of SARS-CoV-2 Spike Protein Inhibitor

The interaction effects of SO-CWB and rosmarinic acid with the SARS-CoV-2 Spike protein were determined using the ACROBiosystem colorimetric assay kit. This *in vitro* assay is a competitive screening in which the tested samples compete with the Spike protein for the binding site on a pre-coated human ACE2 protein. This screening was performed for the wild-type (WT) Spike variant (EP-107).

SO-CWB and rosmarinic acid were tested using the proteins provided in the kit, following the protocol, without preincubation. The concentrations tested were 1.0 to 4.0 mg·mL⁻¹ for SO-CWB and 0.1 to 0.4 mg·mL⁻¹ for rosmarinic acid. A binding between ACE2 and the Spike protein was observed, with values above 90%, indicating no inhibitory effect of the extract or the tested standard, as shown in Figure 1.

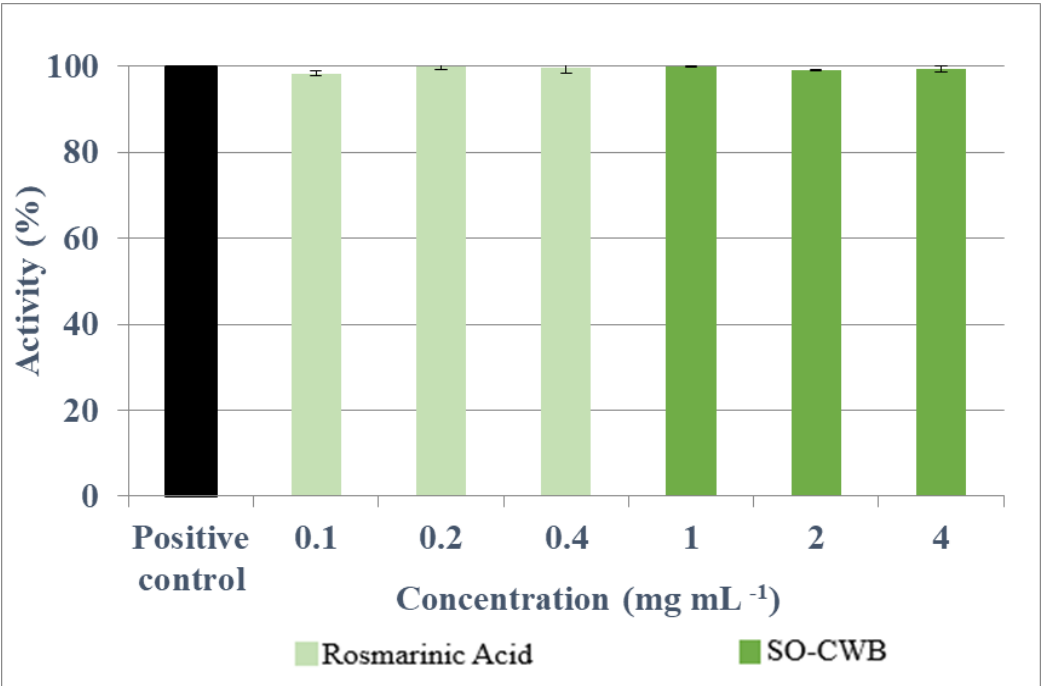


Figure 1. Inhibitory capacity of Spike-ACE2 binding of sage extract and rosmarinic acid with ACE2 protein and HRP-SARS-CoV-2 Spike RBD WT variant.

To evaluate the interaction of the extract and standard separately with the Spike protein, the assay was performed with pre-incubation. This modification aimed to evaluate the individual interactions of the samples with the Spike protein, as molecular docking studies indicate favorable interactions of the compounds with specific regions of the virus [32]. Thus, pre-incubating the extract and standard with the HRP-SARS-CoV-2 Spike RBD provided by the kit (EP-107) aims to evaluate the interaction of this viral region with the extract or rosmarinic acid.

The results obtained with 30 minutes of preincubation with HRP-SARS-CoV-2 Spike RBD are shown in Figure 2(A and B). Figure 2(A) showed that even with preincubation, the SO extract did not interfere with the binding activity between ACE2 and HRP-SARS-CoV-2 Spike RBD (over 90% of activity). Similarly, rosmarinic acid did not interfere, as the result was the same as shown in Fig. 1 without a pre-incubation step.

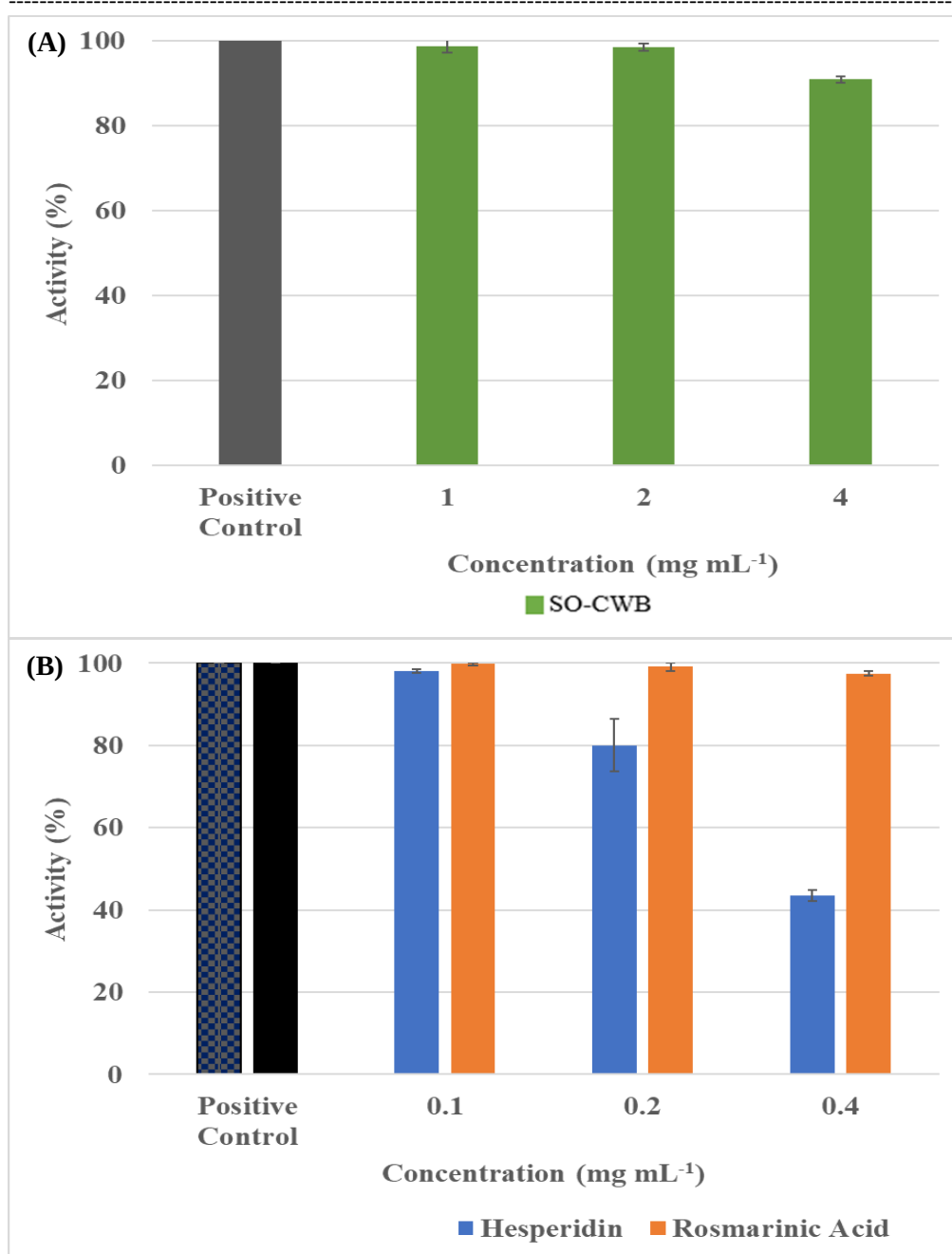


Figure 2. Inhibitory capacity of Spike-ACE2 binding of SO-CWB (A), hesperidin and rosmarinic acid (B) with HRP-SARS-CoV-2 Spike RBD with pre-incubation for 30'.

By contrast, when hesperidin was pre-incubated with HPR-SARS-CoV-2 Spike RBD, binding activity between the Spike protein and ACE2 decreased by 42.6% at a hesperidin concentration of 0.4 mg·mL⁻¹, as shown in Figure 2(B). This finding suggests that hesperidin may interfere with protein binding, potentially blocking Spike-ACE2 interaction in the WT variant.

According to molecular docking studies, rosmarinic acid and hesperidin have shown potential against SARS-CoV-2 by inhibiting viral entry through different mechanisms [5,32]. However, the results of the present study suggest that rosmarinic acid may not act directly on the Spike protein, as previously proposed by docking studies. Instead, rosmarinic acid may affect other pathways involved in SARS-CoV-2 infection. For example, it was reported that rosmarinic acid inhibits the viral 3-chymotrypsin-like protease (3CLpro), a non-structural protein essential for viral replication, with an IC₅₀ value of 6.84 μM [33]. Understanding which

viral proteins are susceptible to inhibition is important for identifying new therapeutic strategies for COVID-19 [33].

In the case of hesperidin, our results showed that this compound interferes with binding between the Spike protein and ACE2, as reported in other studies [31,34]. In addition, Experiments using Vero E6 cells confirmed that hesperidin inhibits the binding between the Spike protein and ACE2 [34]. Molecular docking studies also showed that hesperidin can bind to transmembrane serine protease 2 (TMPRSS2) and angiotensin-converting enzyme 2 (ACE2) [31].

Although rosmarinic acid did not directly block the Spike-ACE2 interaction in this study, this does not exclude the potential of sage extract to interfere with SARS-CoV-2 infection through other mechanisms, such as inhibition of 3CLpro and Mpro proteins. In addition, the extract's strong antioxidant activity may provide complementary benefits by reducing oxidative stress and tissue damage caused by viral infection [27, 35].

4. CONCLUSION

The sage extract prepared in this study contained high levels of secondary metabolites, including phenolic compounds and flavonoids. These classes of compounds are known for their antioxidant activity. Indeed, the extract demonstrated remarkable antioxidant capacity in the FRAP, DPPH, and ABTS assays, indicating its potential as a free-radical-scavenging agent.

However, despite the observed antioxidant activity, the extract and rosmarinic acid did not inhibit binding between the SARS-CoV-2 Spike protein and the ACE2 receptor in an *in vitro* assay, indicating that docking predictions for rosmarinic acid on Spike were ineffective in the *in vitro* assay. These results suggest that although the extract may inhibit ROS production and oxidative stress, it does not interfere with the specific viral interaction mechanism mediated by the Spike protein.

Nonetheless, these findings remain significant, as they highlight the importance of evaluating multiple biological activities when investigating medicinal plant extracts as therapeutic candidates.

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DISCLOSURE STATEMENT

All authors declare that they have no conflicts of interest.

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