Anti-SARS-CoV-2 and Antioxidant Activities of The Methanolic Extract and Its Fractions of Marrubium alysson L. Aerial Parts

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ABSTRACT

The pandemic novel coronavirus disease 2019 (COVID-19) has resulted in enormous economic damage and an exclusive health crisis, so developing a cure for this respiratory infectious disease has proceeded forward at full speed. Natural products and herbal medicines with accepted safety profiles are considered promising sources for discovering new medication leads. Genus Marrubium was reported to exhibit potential antiviral and antioxidant activities. Thus, this study aims to evaluate the inhibitory activity of the total methanolic extract and its soluble fractions: (petroleum ether, methylene chloride, and ethyl acetate) of the aerial parts of Marrubium alysson L. against SARS-CoV-2 main protease (Mpro). Also, they were quantitatively assayed for their phenolics and flavonoids contents, in addition to evaluating their antioxidant activity. The results showed that the petroleum ether fraction has the highest inhibitory activity against SARS-CoV-2 main protease (Mpro) enzyme with IC50 value of 3.959±0.17 µg/mL compared to the reference tipranavir (IC50 1.89±0.08 µg/mL). The ethyl acetate fraction showed the highest total phenolics and flavonoids contents as well as the highest antioxidant activity (IC50 33.81±0.15 µg/mL) compared to the reference L-ascorbic acid (IC50 30.43±0.14 µg/mL). It could be concluded that soluble fractions of the total methanolic extract of Marrubium alysson L. may be used as an alternative source for developing new natural inhibitors against the main protease enzyme and may be suggested for the treatment of COVID-19.

Keywords: Marrubium alysson L., Anti-SARS-CoV-2, ABTS antioxidant assay, total phenolics, flavonoids.

1. INTRODUCTION

Marrubium alysson L. belongs to Genus Marrubium (family Lamiaceae). The Genus Marrubium is a genus of flowering plants, which contains about 40 species. It is located mainly in the temperate regions of the Eurasian Continent and along the Mediterranean Sea. Marrubium species as Marrubium vulgare have a long history of being used in folk medicine for conditions like fever, skin injuries, inflammatory processes, diseases of the digestive, respiratory, and kidney systems, and as a hepatoprotective. Plants belonging to the genus Marrubium are famous for their contents of labdane diterpenes, phenylpropanoids, flavonoids, and essential oils. Marrubium alysson L. plant is a perennial herb with round, cuneate-base, coarsely crenate and fleece-covered leaves, pink or bluish corolla, and fruit-calyx with five spreading star-shaped spines. Previous studies reported that its extracts had...
anti-inflammatory, antioxidant, analgesic, hypoglycaemic, and antimicrobial activities.\textsuperscript{13-15}

Coronavirus 2 (SARS-CoV-2), a novel strain of the coronavirus family that causes severe acute respiratory syndrome, was the cause of the COVID-19 sickness. It is extremely infectious, pathogenic and has spread rapidly throughout the world, so the World Health Organization (WHO) designated it a pandemic on March 11, 2020. Despite the fact that over hundreds of clinical trials and preclinical studies have been conducted to find treatments against COVID-19, up to date, there is no effective treatment for this disease.\textsuperscript{16,17} The SARS-CoV-2 main protease (M\textsuperscript{pro}) enzyme, also known as the chymotrypsin-like protease 3CLpro, is responsible for the release of vital functional peptides during proteolysis, which is crucial to coronavirus replication and the subsequent progression of the virus life cycle.\textsuperscript{18} It has been recognized as a significant target for treatment approaches.

Phenolic compounds and flavonoids are considered effective antioxidants owing to their ability to neutralize the free radicals that cause oxidative stress.\textsuperscript{19-21} Although synthetic anti-oxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are very efficient, they also have several negative side effects, including the development of tumors when used at high dosages and over an extended period of time. As a result, there is growing interest in utilizing naturally occurring antioxidants instead of synthetic antioxidants in food, cosmetic, and pharmaceutical goods.\textsuperscript{22}

Plant-derived natural products with therapeutic relevance have recently gained a lot of interest in exploring highly effective natural drugs for several diseases because these products are available and relatively safe. It was reported that several species of Marrubium, such as M. peregrinum and M. deserti showed antiviral activity against different viruses, including coronaviruses.\textsuperscript{17,23,24} This encouraged us to evaluate Marrubium alysson L. plant growing in Egypt as anti-COVID-19.

This study evaluates the antioxidant and anti-SARS-CoV-2 activities of the total methanolic extract and its soluble fractions of Marrubium alysson L. aerial parts to discover potential antioxidant and anti-COVID-19 therapeutics. Moreover, their total phenolics and total flavonoid contents were quantitatively estimated.

It is worth noting that this study is the first one to report the inhibitory activity of the total methanolic extract and its soluble fractions of Marrubium alysson L. aerial parts against the SARS-CoV-2 main protease (M\textsuperscript{pro}) enzyme.

2. MATERIAL AND METHODS

2.1. Apparatus and Reagents

Rotary flash evaporator (Büchi, Switzerland), Speckol 11 (Carl Zeiss-Jena) spectrophotometer, ELx800UV (USA), and a fluorescent microtiter plate reader. Folin-Ciocalteu reagent, aluminium chloride reagent, Gallic acid (Sigma, 98%), Quercetin (Sigma, 95%), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) reagent. Inhibition of M\textsuperscript{pro} enzyme activity was measured using the Fluorogenic M\textsuperscript{pro} Assay Kit (Catalog #79955-1); the kit includes purified main protease and is available in a convenient 96-well test plate, main protease assay buffer and fluorogenic substrate.

2.2. Plant material

The aerial parts of Marrubium alysson L. were collected from North Coast at the Mediterranean coastal strip, Egypt, in April 2019. The plant was authenticated by Prof. Ibrahim Mashaly, Professor of Ecology, Faculty of Science, Mansoura University, Egypt. Voucher specimens of the plant have been kept in the Pharmacognosy Department of the Faculty of Pharmacy at Mansoura University in Egypt and were coded as MA-2-2019.

2.3. Preparation of the extracts:

The aerial parts of Marrubium alysson L. were ground into a fine powder after being air-dried at room temperature in the shade. Extraction was done by macerating the powdered, air-dried aerial parts (2 Kg) in 70% methanol at room temperature (7 x 3.5 L). The dark green viscous residue (220 g) was produced after the collected methanolic extract was evaporated under reduced pressure and allowed to dry in a desiccator over anhydrous CaCl\textsubscript{2}. The dried methanolic residue (200 g) was dissolved in the least amount of methanol, diluted with a suitable volume of distilled water, and fractionated with petroleum ether 60/80, methylene chloride, and ethyl acetate. Each time, the solvent was dried under reduced pressure and allowed to dry in a desiccator over anhydrous CaCl\textsubscript{2}. The dark green viscous residue (220 g) was produced after the collected methanolic extract was evaporated under reduced pressure and allowed to dry in a desiccator over anhydrous CaCl\textsubscript{2}. The dried methanolic residue (200 g) was dissolved in the least amount of methanol, diluted with a suitable volume of distilled water, and fractionated with petroleum ether 60/80, methylene chloride, and ethyl acetate. Each time, the solvent was dried under reduced pressure, resulting in petroleum ether soluble fraction (35.34 g), methylene chloride soluble fraction (30.23 g), ethyl acetate soluble fraction (13.47 g), and aqueous fraction (111.24 g).

2.4. Biological activities

2.4.1. Fluorogenic substrate assay

Based on the fluorogenic substrate assay,\textsuperscript{25,26} the SARS-CoV-2 main protease (M\textsuperscript{pro} or 3CL\textsuperscript{pro}) enzyme's inhibiting activity has been examined. The principle based on the peptide
substrate's C-terminal is connected to a fluorophore (Edans), and a peptide substrate's N-terminal fluorescence quencher (Dabcyl) inhibits the fluorescence signal of Edans. The peptide substrate has low fluorescence as the Dabcyl in the N-terminal quenching the intensity of Edans' fluorescence in the C-terminal. The substrate was hydrolyzed by the $M^{Pr}$, yielding the non-fluorescent Dabcyl fragment and the intensely fluorescent Edans fragment. As a result, the fluorescence signal has increased in proportion to the protease activity. The release of fluorescent fragments is inhibited by the main protease ($M^{Pr}$) inhibitor, which reduces the intensity of the fluorescent signal. A fluorescent microtiter plate reader that can read excitation/emission at 360/460 nm is used to measure the intensity of the fluorescence.

### 2.4.2. Total phenolics determination assay

Using gallic acid as a standard, total phenolics were determined using Folin-Ciocalteu colorimetric method.\(^{27}\) One milligram of the total methanolic extract and its soluble fractions were dissolved in 1 mL of MeOH, then 4 mL of sodium carbonate (7% w/v) were added and mixed completely with 5 mL of Folin-Ciocalteu regent (10% v/v), the blue mixture was kept for 30 minutes in a water bath at 40°C. Then, against a blank (1 mL of methanol was mixed with 4 mL of sodium carbonate (7% w/v) and 5 mL of Folin-Ciocalteu regent (10% v/v) to create a final volume of 10 mL), the absorbance at 760 nm was measured, every measurement was made in triplicate. The absorbance values at various gallic acid concentrations from (0-1 mg/mL) that were previously prepared in the same manner for the quantitative estimation of phenolics content were used to plot the calibration curve. Phenolics content was expressed as milligrams per gram of gallic acid equivalent (GAE).

### 2.4.3. Total flavonoids determination assay

Using quercetin as a reference substance, the total flavonoids content of each extract was calculated spectrophotometrically by the aluminum chloride method.\(^{28}\) This technique is based on the presence of a yellow compound made of flavonoids-aluminum that have absorbptivity at around $\lambda$ 415 nm. 10 mg of the total methanolic extract and its fractions was dissolved in 1 mL of ethanol, then 100 $\mu$L of plant extract in ethanol was combined with 100 $\mu$L of 20% AlCl$_3$ in ethanol and 50 $\mu$L of acetic acid, then diluted with ethanol to 5.0 mL, the intensity of the formed yellow color was estimated at 415 nm and were measured after 40 min. Against control samples (100 $\mu$L of plant extract and 50 $\mu$L of acetic acid, then diluted with ethanol to 5.0 mL), under the same circumstances, the absorption of a standard quercetin solution of different concentrations from (0-1 mg/mL) in ethanol was evaluated, every measurement was made in triplicate, and a standard curve was produced. Flavonoids content was expressed as milligrams per gram of quercetin equivalent (QE).

### 2.4.4. Free radical scavenging activity (ABTS anti-oxidant assay)

Determination of ABTS Radical Scavenging Activity was carried out as reported.\(^{29,30}\) Preparing the ABTS\(^+\) radical cation (blue-dark green) by adding equal amounts of ABTS (2,2' -Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) stock solution (colorless; 7 mM in pure distilled H$_2$O) and potassium persulfate stock solution (K$_2$S$_2$O$_8$: 3.5 mM in pure distilled H$_2$O), the mixture was kept and left to stand in the dark at room temperature overnight (for 12–16 hours) till the reaction was completed, then the ABTS\(^+\) working solution was prepared by diluting the ABTS\(^+\) stock solution in pure EtOH until it had an absorbance ($A_{blank}$) of 0.7±0.02 at a wavelength of 734 nm. The solution was then equilibrated with a temperature control set at 30°C in an incubator ($A_{blank}$).

The free radical scavenging activity was evaluated by combining 1.5 mL of the blue-green ABTS\(^+\) working solution with varied concentrations ranging from 10 to 100 $\mu$g (in distilled H$_2$O, pure EtOH, or a mixture of both of them according to the solubility) of all test extract and its fractions. After the mixing, the change in absorbance at 734 nm for each test extract ($A_{test}$) was taken after 15 minutes of their mixing because the steady state was reached after that time in the current experiment. Values are the averages of three separate calculations (as all the measurements were taken three times). The following equation was used to compute the percent decrease in absorbance, which represents the test extract's ability to scavenge ABTS\(^+\) radical cations:

\[
\% \text{ inhibition} = 100 \left( \frac{A_{blank} - A_{test}}{A_{blank}} \right)
\]

After 15 minutes of reaction, the inhibitory concentration (IC$_{50}$) of the total methanolic extract and its fractions were calculated and compared to that of L-ascorbic acid (taken as the reference and standard antioxidant compound in this assay). The IC$_{50}$ is the concentration of any test extract required to reduce the absorption or amount of ABTS\(^+\) radical cations by 50% at a wavelength of 734 nm. Using the GraphPad Prism 6 software (U.S.A., 2015), the antioxidant or anti-ABTS\(^+\) IC$_{50}$ value (for each test compound and the reference L-ascorbic acid) was computed. The lower the IC$_{50}$ value, the more potent the test extract as an antioxidant is (i.e., the stronger the antioxidant activity of the test extract is).

### 3. RESULTS AND DISCUSSION OF BIOLOGICAL ACTIVITIES
3.1. Anti-SARS-COV-2 activity (main protease (M\text{pro}) inhibitory activity)

COVID-19 is regarded as a severe public health danger throughout the world as it is an extremely contagious disease, pathogenic, and can spread swiftly. Different strains of this virus have evolved over the past years, leading to increased transmissibility and severity of the disease. Since there are no specific medications to treat COVID-19, it has become vital to develop and produce anti-COVID-19 medications. Natural products and their derivatives have been reliable sources to cure a wide range of illnesses for a very long time in human history. Therefore, the evaluation of anti-SARS-CoV-2 activity is very important. The anti-SARS-CoV-2 of the total methanolic extract and its fractions of the aerial parts of Marrubium alysson L. were examined. The total methanolic extract exhibited weak inhibitory activity against SARS-CoV-2 main protease (M\text{pro}) enzyme (IC\text{50} 73.89±3.14 µg/mL) in comparison with the reference antiviral compound Tipranavir (IC\text{50} 1.89±0.08 µg/mL), which may be attributed to the antagonistic effects of different classes of components as terpenes, flavonoids and phenylpropanoids with each other. However, the petroleum ether fraction demonstrated strong inhibitory activity against SARS-CoV-2 main protease (M\text{pro}) enzyme (IC\text{50} 3.959±0.17 µg/mL), which may be attributed to the synergistic effect of its components as fatty acids, sterol, and terpenes. Moreover, the methylene chloride and ethyl acetate fractions showed moderate inhibitory activity against SARS-CoV-2 main protease (M\text{pro}) enzyme with IC\text{50} (11.62±0.49, and 16.1±0.68 µg/mL, respectively) (Table 1). Our findings shed light on the potential use of the Marrubium alysson L. plant as a green source of M\text{pro} inhibitors.

Table 1: Results of the M\text{pro} inhibitory activity of the total methanolic extract of the aerial parts of Marrubium alysson L. and its fractions compared with the standard Tipranavir.

<table>
<thead>
<tr>
<th>Extract/fractions</th>
<th>In vitro COV-M pro IC\text{50} (µg/mL)±S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tipranavir (Standard)</td>
<td>1.89±0.08</td>
</tr>
<tr>
<td>Methanolic extract</td>
<td>73.89±3.14</td>
</tr>
<tr>
<td>Petroleum ether fraction</td>
<td>3.959±0.17</td>
</tr>
<tr>
<td>Methylene fraction</td>
<td>11.62±0.49</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>16.1±0.68</td>
</tr>
</tbody>
</table>

*Tipranavir is used as standard with IC\text{50} 1.89±0.08 µg/mL.

3.2. Total phenolics determination assay

Quantitative analysis by UV-visible spectrophotometer after establishing calibration curves with different concentrations of gallic acid. Results shown in (Table 2) revealed that the total methanolic extract of aerial parts of Marrubium alysson L. possessed moderate phenolics content. The fractions' results revealed that the ethyl acetate fraction possessed the highest total phenolics content; 226.7 mg /g (GAE), followed by the methylene chloride fraction; 90.5 mg /g (GAE). Finally, petroleum ether fraction possessed the lowest phenolics content, 45.59 mg /g (GAE) (Figure 1, Table 2).

Table 2: The total phenolics contents of total methanolic extract of the aerial parts of M. alysson L. and its fractions.

<table>
<thead>
<tr>
<th>Extract/fractions</th>
<th>mg /g (GAE)±S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract</td>
<td>73.36±1.73</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>45.59±1.50</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>90.50±1.74</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>226.70±4.23</td>
</tr>
</tbody>
</table>

3.3. Total flavonoids determination assay

Quantitative analysis by UV-visible spectrophotometer after establishing the calibration curve with different concentrations of quercetin. Results are shown in (Table 3) which revealed that the total methanolic extract of the aerial parts of Marrubium alysson L. possessed moderate flavonoid content. The fractions' results revealed that the ethyl acetate fraction possessed the highest total flavonoid content; 316.80 mg /g (QE), followed by the methylene chloride fraction;
200.5 mg/g (QE). The Petroleum ether fraction possessed the lowest flavonoid content, 79.54 mg/g (QE) (Figure 2, Table 3).

Table 3: The total flavonoid content of the total methanolic extract of the aerial parts of *M. alysson* L. and its fractions.

<table>
<thead>
<tr>
<th>Extract/fractions</th>
<th>mg/g (QE)±S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract</td>
<td>211.40±1.48</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>79.54±1.31</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>200.50±2.52</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>316.80±4.78</td>
</tr>
</tbody>
</table>

3.4. Free radical scavenging activity (ABTS antioxidant assay)

Numerous antioxidant defense mechanisms in the body provide resistance against free radicals. As a result, oxidative stress results from an imbalance between the body’s antioxidant defense system and reactive oxygen species (ROS) production. Polyphenolic compounds and flavonoids in plants are responsible for their antioxidant activity. Polyphenols have the ability to scavenge reactive oxygen species that can seriously harm a person’s health as these reactive oxygen species play a role in many different diseases, such as cancer, atherosclerosis, and cardiovascular disorders. Therefore, the evaluation of antioxidant activity is very important.

The antioxidant activity of the total methanolic extract of the aerial parts of *Marrubium alysson* L. and its fractions were evaluated using the ABTS method. Results shown in (Table 4) revealed that the ethyl acetate fraction had the greatest radical scavenging activity among the other examined fractions with an IC50 value of 33.81±0.15 µg/mL compared to standard ascorbic acid with an IC50 value of 30.43±0.14. This could be explained by the ethyl acetate fraction’s high polyphenols content and high flavonoid content, which are crucial to the observed antioxidant activity. It was followed by total methanolic extract with IC50 79.17±0.35, the methylene chloride fraction and petroleum ether fraction that showed almost the same IC50 value 82.48±0.44 µg/mL, 84.44±0.42 µg/mL respectively that considered moderate radical scavenging activity compared to standard ascorbic acid (Table 4).

Table 4: Antioxidant activity (IC50, µg/mL) of total methanolic extract and its fractions of *Marrubium alysson* L. using ABTS antioxidant assay.

<table>
<thead>
<tr>
<th>Extract/ Fractions</th>
<th>ABTS IC50 (µg/mL)±S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid* (Standard)</td>
<td>30.43±0.14</td>
</tr>
<tr>
<td>Total methanolic</td>
<td>79.17±0.35</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>84.44±0.42</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>82.48±0.44</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>33.81±0.15</td>
</tr>
</tbody>
</table>

*Ascorbic acid (ABTS) is used as a standard antioxidant with IC50 30.43±0.14 µg/mL.

From the results listed in (Tables 2,4) and (Figure 3), we can conclude the following:

The antioxidant activity of the total methanolic extract and its fractions is mainly attributed to their polyphenols content, e.g., the ethyl acetate extract had the highest polyphenols content and, subsequently, the lowest IC50, which
means the highest antioxidant activity. We can conclude that
the phenolics content plays a great role in the antioxidant
activity, but the antioxidant activity is not restricted to
phenolics compounds and may be related to other types of
compounds; the evidence for that is petroleum ether fraction
showed moderate antioxidant activity while their phenolics
content was very low.

4. CONCLUSION:

Evaluation of the anti-SARS-CoV-2 and antioxidant activities
of the total methanolic extract and different soluble fractions of
Marrubium alysson L. aerial parts were carried out in order to
find and explore highly effective natural main protease
inhibitors and antioxidant agents. This study indicated that the
petroleum ether fraction had the highest inhibitory activity
against SARS-CoV-2 main protease (M^Pr). The ethyl acetate
fraction with the highest level of total phenolics and
flavonoids showed the highest antioxidant activity. Our result
was a preliminary screening for using Marrubium alysson L.
plant as an anti-COVID-19 and antioxidant drug, and further
phytochemical investigation may be required.

CONFLICT OF INTEREST:

The authors confirm that the article content has no conflicts
of interest.

5. REFERENCES


