



Research Article

# Modern *In Silico* Molecular Docking Perspective for Investigation of *Rindera lanata* Bunge var. *lanata* Targeting: Phytochemical Profile, Phytotoxicity and Bioactivity Assays

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## Abstract

**Background:** Finding herbal herbicides with biodegradability and selectivity against weeds is an interesting and vital challenge for human health. The study was considered to analyze the phytochemical profile, phytotoxicity and biological activities of *Rindera lanata* together with a molecular docking perspective.

**Methods:** Aerial parts of *R. lanata* were successively solvent extracted with the Soxhlet apparatus, followed by maceration. Different chromatography methods were employed to isolate and purify the major compounds of the plant. The compounds were assigned by spectroscopic methods, containing 1D and 2D NMR (Nuclear Magnetic Resonance). Several in vitro antioxidant assays, together with an antimicrobial assay, alongside the allelopathic property on the germination of *Cuscuta campestris* seeds, were assessed upon the extracts. As a final point, molecular docking was conducted to evaluate the interactions among the identified compounds with 4-hydroxyphenyl pyruvate dioxygenase (HPPD), glutamine synthetase (GS), and acetohydroxyacid synthetase (AHAS).

**Results:** Phytochemical analysis of the extracts led to the identification of a new flavonoid 3, 5, 4'-tri-O-methylquercetin (1), apigenin (2), chrysoeriol (3), luteolin-7-O-glucoside (4) and rutin (5). It was exposed that ethyl acetate extract not only illustrated the highest antioxidant effect in all the in vitro assays but also exhibited the maximum antibacterial effect against *Staphylococcus epidermidis*. Effectively, it showed conventional allelopathic activity in preventing germination, seedling length and seedling weight growth of *C. campestris* seeds, as well as a concentration-dependent enhancement ( $p \leq 0.05$ ). Docking analysis results indicated that rutin had a good affinity for HPPD, GS and AHAS enzymes, with docking score values of -11.454, -10.422 and -10.152 kJ/mol, respectively.

**Conclusion:** The present study suggested that isolated flavonoids from *R. lanata* could be used as the lead compounds in the development of natural herbicides for strong bonds in the active region of HPPD, GS and AHAS enzymes.

## Introduction

In recent decades, one of the hazardous challenges threatening human health has been the contamination of plant and animal food sources with dangerous industrial herbicides.<sup>1,2</sup> Pollution of the soil and underground water

and multisystem defects in humans are among the problems associated with long-term contact with some industrial herbicides.<sup>3</sup> As regards, the use of bioherbicides from plant sources with phytotoxic effects on weeds has received attention. The advantages of these herbal herbicides are

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their biodegradability and selective mechanism, shorter half-life, and lack of adverse consequences.<sup>4-6</sup>

As we known, allelopathy effect is a biochemical communication between a plant or microorganism with other biological systems (such as plants, fungi, bacteria, etc.) that affects their growth and development. In an allelopathic process, a plant produces secondary metabolites (allelochemicals) that are harmful to the growing of its neighbors.<sup>7</sup> Plants allelochemicals are released in the soil as volatile or non-volatile organic compounds and directly or indirectly affect the germination of other plants. Besides, allelochemicals act as a plant's defense mechanism against weeds, insects, fungi, bacteria, etc.<sup>8</sup> Many plants, including the family Boraginaceae, produce allelopathic compounds in their leaves, seeds, stems, roots, and flower organs.<sup>9,10</sup> The popularity of allelochemicals for use as biological herbicides is enhancing daily due to their economic efficiency, biodegradability and compatibility with the environment.<sup>11</sup>

The inherent toxicity of herbal medicinal products is caused by plant secondary metabolites with toxic potential and the plant's growth environment.<sup>12</sup> However, plant toxicity is not always considered as a bad characteristic; for example, phytotoxicity and allelopathic potential are useful in fighting weeds today.<sup>13</sup>

The genus *Rindera* belonging to the Boraginaceae family, officially contains 25 accepted species and 5 taxa, mainly in central and western Asia and eastern Europe.<sup>14,15</sup> *Rindera lanata* Bunge var. *lanata* is a recognized species, synonymous with *Cynoglossum lanatum* Lam., *Cyphomattia lanata* Boiss. and *Mattia lanata* (Lam.) Roem. & Schult. species.<sup>16-18</sup>

Anti-inflammatory properties are among the reported traditional uses of plants from *Rindera* genus.<sup>19</sup> Moreover, the aerial parts essential oils of the of *R. lanata* var. *canescens* have shown antibacterial activity.<sup>20</sup> In addition, the methanolic extract of *R. lanata* var. *lanata* demonstrated good antiviral activity against human rotavirus.<sup>19</sup> In a recent study, *n*-hexane and methanolic extracts obtained from in vitro-cultivated shoots and hairy roots of *R. graeca* indicated substantial anti-proliferative effects against lung cancer cell lines.<sup>21</sup>

Analysis of the *R. oblongifolia* seed oil led to the identification of triglyceride acids; five different types of monoenoic acids were identified in the length of the carbon chain but with the same carbon terminal.<sup>22</sup> In another study, the lipid content of the fruit oil of *R. oblongifolia* was investigated. Total lipid contents included neutral lipids, glycerolipids, phospholipids and free fatty acids.<sup>23</sup> Recently, rinderidine and oblongifolidine, two new alkaloid compounds, were found in aerial parts ethanolic extract of the *R. oblongifolia*.<sup>24</sup> In the viewpoint of phytochemicals, echinatine (an alkaloid) was reported in *R. echinata*.<sup>25</sup> Elsewhere, numerous studies conducted on variant organs of *Rindera umbellata* showed that this plant was a rich source of triglyceride fatty acids and pyrrolizidine alkaloids.<sup>26,27</sup> In another survey, the

contents of the *R. lanata* var. *canescens* essential oils achieved with two steam distillation methods (HD) and microwave (MW) were analyzed. Aldehydes and alcoholic hydrocarbons were the major classes of components in the MW and HD essential oils, respectively.<sup>20</sup> The methanolic extract content of the *R. lanata* var. *lanata* leaves was assigned consuming the liquid chromatography-mass spectrometry (LC-MS/MS). The identified compounds were: quinic acid, chlorogenic acid, protocatechuic acid, malic acid, caffeic acid, rosmarinic acid, *p*-coumaric acid and five flavonoids named rutin, hesperidin, hyperoside, apigenin and rhamnetin.<sup>19</sup> In the phytochemical study carried out on the methanolic extract of the leaf of *Rindera graeca*, an endemic plant of Greece, using LC-MS technique with HPLC-ESI-TOF-MS apparatus, it was revealed that the major phenolic compounds were caffeic acid, salvianolic acid A, rhabdosiin disodium salt, rhabdosiin and salvianolic acid B and chlorogenic acid and pyrrolizidine alkaloids of echinatine, echinatine N-oxide and rinderine N-oxide were also identified. Additionally, in that study, quercetin-3-rutinoside-7-rhamnoside, rutin and rosmarinic acid metabolites were isolated using column chromatography and preparative thin layer chromatography (PTLC).<sup>28</sup> Recently, numerous studies have been conducted to develop the biosynthesis potential of secondary metabolites with the technique of *in vitro* hairy (transgenic) roots on *R. graeca* culture, in which the amount of medicinal bioactive compounds produced by the plant such as naphthoquinone, lithospermic acid B and rosmarinic acid has been significantly increased.<sup>29-32</sup>

Considering the great phytochemical potential and numerous biological properties reported from the *Rindera* genus plus limited studies on the *R. lanata* var. *lanata* species, the current study designed to investigate the phytochemical profile, allelopathic phytotoxicity, antimicrobial and antioxidant potential of *R. lanata* different extracts, along with the use of a modern *in silico* molecular docking interaction technique between the active components of the extract (ligand) and selected proteins (receptor).

## Methods

### General experimental procedures

Chloroform, *n*-hexane, ethyl acetate, potassium acetate, sodium acetate, aluminum chloride, dimethyl sulfoxide (DMSO), potassium ferricyanide ( $K_3[Fe(CN)_6]$ ), sodium carbonate, gallic acid, Folin-ciocalteu reagent, acetic acid, formic acid, ferric chloride ( $FeCl_3$ ), trichloroacetic acid (TCA), sodium chloride, potassium chloride, monopotassium phosphate, hydrogen peroxide, disodium phosphate, ascorbic acid,  $\beta$ -carotene, linoleic acid, silica gel 70-230 mesh and 230-400 mesh and sulfuric acid that were all obtained from Merck (Darmstadt, Germany). Sigma-Aldrich (Steinheim, Germany) provided the quercetin and 2,2-diphenyl-1-picrylhydrazyl (DPPH), while the companies Kian Kaveh (Iran), Samchun (Korea), and Ghazalshimi (Iran) supplied the *n*-butanol, methanol,

and trifluralin EC48% (w/v), respectively. The Mueller-Hinton agar (MHA) and the Mueller-Hinton broth (MHB) were purchased from the Canadian company Quelab. The Pasteur Institute of Tehran (Iran) provided the microorganism strains. Additionally, the standard test disks of nystatin, tetracycline and ampicillin were provided by Padtan Teb (Iran).

#### **Plant materials**

The *R. lanata* aerial parts were gathered in September 2019 from the mountains around Tabriz, eastern Azerbaijan Province, Iran. The plant botanical identification was conducted by Professor Hossein Nazemiyah. A voucher specimen (TBZFPH 4100) was deposited in the herbarium of faculty of pharmacy Tabriz university of medical sciences. Subsequently, the plant was air-dried away from sunbeam and powdered for further analysis.

#### **Solvent extractions and fractionations**

The *R. lanata* powder (200 g) was solvent extracted via a Soxhlet apparatus with *n*-hexane and chloroform, separately (1.3 L: each) at a temperature (>75°C) for 8 hours until the solvent in the last siphonage was colorless. Following that, remaining plant material was extracted with 70% ethanol (3 x 4 L) at room temperature (25°C) in 3 days by the maceration technique. The obtained solutions were dried out at 40°C upon vacuum via a rotary evaporator (Heidolf, Germany). Then, the hydroalcoholic dried powder was suspended in 100 mL of distilled water and the liquid-liquid extraction was conducted with ethyl acetate and then with *n*-butanol (the same volume).<sup>33</sup> It is worth noting that all decantation steps were performed at a temperature of 25°C. The contact time between the two organic and aqueous phases was 3-5 hours. In addition, the decantation continued until the organic phase became completely transparent (6-9 times). Subsequently, the obtained ethyl acetate, *n*-butanol and aqueous fractions were dried under vacuum at a low temperature and kept at -8°C until the next usage.

#### **Extraction of the lipophilic content from *R. lanata* *n*-hexane extract**

The decantation method was used to extract the lipophilic contents of the *n*-hexane extract. In this way, at room temperature (25°C), 200 mg of dried *n*-hexane fragment was dissolved in 10 mL of *n*-hexane and then subdivided with the same volume of 90% methanol via a decantation funnel. The contact time between the two organic and aqueous phases was 1 hour and the number of decantation times was 4 times until the methanol phase was completely transparent. After drying the *n*-hexane phase, it was used for as chromatography-mass spectroscopy (GC-MS) study.<sup>34</sup>

#### **GC-MS identification of the lipophilic content**

Screening of the lipophilic content of the *R. lanata* *n*-hexane extract was performed by GC-MS with a Shimadzu GC-

MS-QP5050A apparatus with a methylphenylsiloxane (DB-1) capillary column (60 m x 0.25 mm; film thickness 0.25 µm). Helium was used as the carrier gas throughout the inspections, which were carried out at a linear velocity of 29.2 cm/s and a split ratio of 1:24 with a flow rate of 1.3 mL/min. The ionization source and the contact were heated to 270 and 280 °C, respectively. The initial temperature in the main oven was 60 °C, and it was raised by 8 °C every minute to a steady 290 °C for 3 minutes. An MS detector was used to identify the ionized compounds, with settings comprising an ionization voltage of 70 eV, an ion chamber temperature of 250°C, and a scan mass range of 35 to 456 amu in 0.4 s. Standards of *n*-alkanes (C<sub>8</sub>-C<sub>21</sub>) were used upon the same chromatographic instructions. The retention index (RI) of each signal was calculated and their mass spectra were compared with libraries (NIST 107, NIST 21 and WILEY229 libraries). Subsequently, the compounds of the *n*-hexane lipophilic content were identified.<sup>35-37</sup>

#### **Fractionation of the ethyl acetate extract of *R. lanata***

Column chromatography (height 80 cm, diameter 3 cm), with 125 g of silica gel (mesh 70-230), was applied to fractionate 2 grams of ethyl acetate extract. The process of Elution was conducted using gradient solvents from 80% *n*-hexane-ethyl acetate to 100% ethyl acetate. Elution was continued with gradually increasing solvents of methanol, water and acetic acid in several steps until concentration is reached to methanol, water and acetic acid with a ratio of 65-25-10. After controlling the obtained fractions by thin layer chromatography (TLC) and recrystallization, 2 compounds were purified, including: compound **1** (7 mg, separated in elution of *n*-hexane-ethyl acetate at a ratio of 40-60), with an amorphous white powder and compound **2** (6 mg, eluted with methanol-ethyl acetate at ratio of 3-97). The rest of the ethyl acetate fractions were combined dependent on the TLC patterns and the spots were visualized by both UV and anisaldehyde-sulfuric acid staining to create 11 subfractions (*F1-F11*). *F5* (410 mg) was subjected to column chromatography (silica gel mesh 70-230), with isocratic acetic acid-water-methanol-ethyl acetate elution solvent (2-3-5-90), and another pure compound was obtained (**3**, 7 mg). All fractionation steps were performed at 25°C.

#### **Fractionation of *n*-butanol extract of *R. lanata***

*n*-Butanol extract was fractionated using solid-phase extraction (SPE). Two g from the dried *n*-butanol fraction (2 g x 2) was loaded on a Sep-Pak (C<sub>18</sub>, 10 g, 35 mL cartridge) via a gradient elution with a mixture of methanol -water solvents (10-90, 20-80, 40-60, 60-40, 80-20, and 100-0). Each step was conducted with 200 mL of the mentioned solvents and all fractionation steps were conducted at 25°C. Subfractions were dried and kept at -8°C for additional investigation. After a preliminary TLC analysis, fraction of 10% Sep-Pak was picked for further analysis. Preparative thin layer chromatography (PTLC)

was used for extra fractionation with glass plates (20 x 20 cm) with a thick coating (5 mm) of silica gel GF<sub>254</sub>. A total of 320 mg of the fraction of 10% *n*-butanol extract was diluted in the minimum volumes of methanol and loaded on the plates. Once the plates were developed in an ethyl acetate-methanol-water-acetic acid solvent system (mobile phase) at a ratio of 78-10-6-6, they were dried and then the side strip of the plates was visualized with sulfuric acid anisaldehyde reagent. Compounds 4 (7 mg) 5 (6 mg), were parted with R<sub>f</sub> values of 0.82 and 0.45, respectively, and after dissolving in 90% methanol solvent and centrifuging, they were separated from silica gel. Figure 1 shows the process of isolation and purification of compounds from *R. Lanata*.

### Structure elucidation of purified compounds

The structure of the isolated compounds was determined using NMR (nuclear magnetic resonance) via <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, HSQC and HMBC spectra (500 MHz Bruker AVANCE III and 300 MHz Bruker AVANCE II instruments, Germany). Deuterated DMSO was used as the NMR solvent.

### Bioactivity assays (In vitro tests for evaluating antioxidant activeness)

#### DPPH radicals scavenging assay

The radical scavenging activities of chloroform, ethyl acetate, *n*-butanol and aqueous extracts of *R. lanata* were assessed applying the DPPH radical scavenging assay<sup>38</sup> with modifications. For each extract and quercetin as the standard, stock solutions were prepared in methanol and after centrifugation at 16128 G-force, dilutions were prepared. A total of 1250 µL of DPPH solution (0.04 mg/mL) was combined to 75 µL of each dilution and were incubated at room temperature for 30 minutes in the

darkness. Then At a wavelength of 517 nm, the samples were analyzed for their absorbance. The amount of DPPH radical inhibition was determined, and the concentration of the desired extract that reduced 50% of free radicals was measured as RC<sub>50</sub>.

#### Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging assay

The method of Singh *et al.*<sup>39</sup> was utilized to quantify the H<sub>2</sub>O<sub>2</sub> inhibition effect of *R. lanata* (chloroform, ethyl acetate, *n*-butanol and aqueous) extracts. First, a solution containing 50 mM of H<sub>2</sub>O<sub>2</sub> was produced in phosphate buffer (pH=7.4), and then several concentrations of *R. lanata* extracts and an ascorbic acid as the standard (concentration 10-2000 µg/mL) were serially prepared in phosphate buffer. Each dilution was repeated three times. Each of the samples was given a 0.5 mL of the prepared H<sub>2</sub>O<sub>2</sub> solution, and the absorbance of the solutions at 230 nm was measured after 10 minutes of incubation in the dark. The H<sub>2</sub>O<sub>2</sub> inhibition percentage was considered for each sample using the below formula, and IC<sub>50</sub> values were determined for the extracts.

$$\% \text{ Inhibition of H}_2\text{O}_2 = \frac{\text{Absorbance}_{\text{Blank}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{Blank}}} \times 100$$

#### Ferric reducing antioxidant power (FRAP) assay

To evaluate the reducing power of *R. lanata* (chloroform, ethyl acetate, *n*-butanol and aqueous) extracts, the modified method of Tounsi *et al.*,<sup>40</sup> was used. After preparing different concentrations of samples and quercetin as the standard (concentrations from 0 to 2000 µg/mL) in methanol, A volume of 1 mL from each sample was combined with 2.5 mL of phosphate buffer solution at a pH of 6.6, along with an additional 2.5 mL of a potassium ferricyanide solution with a concentration of 1%.

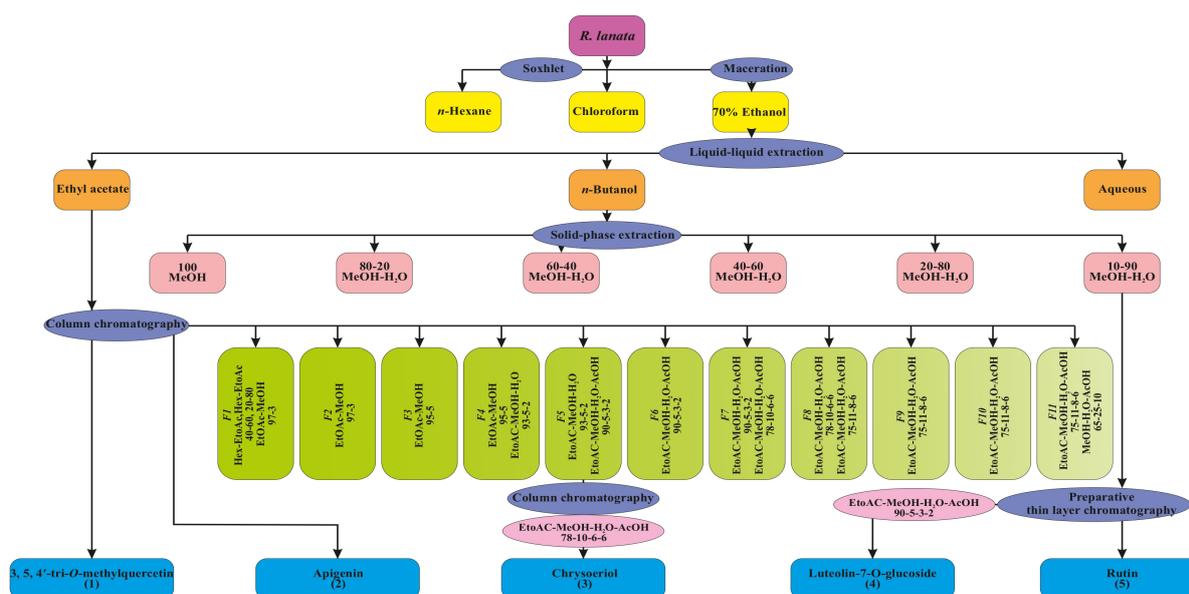


Figure 1. Isolation and purification process of *Rinder lanata* compounds.

subsequently, incubation was performed at 50°C for 20 minutes. After that, 2.5 mL of 10% trichloroacetic acid was added to the mixture, and the solution was centrifuged for 10 minutes at 3000 G-force. On the 2.5 mL of supernatant, 2.5 mL of distilled water and 0.5 mL of a 0.1% ferric chloride solution were added. Spectrophotometrically, the samples absorption rate was calculated at 700 nm. Absorption diagrams were drawn according to concentration, and the EC<sub>50</sub> rate (the concentration of the extract that the absorption value was 0.5) was determined for each sample.

#### *β*-Carotene /linoleic acid bleaching assay

The improved method of Miraliakbari *et al.*<sup>41</sup>, was consumed to determine the *β*-carotene/linoleic acid bleaching power of *R. lanata* (chloroform, ethyl acetate, *n*-butanol and aqueous) extracts.<sup>41</sup> First, in separate tubes, 200  $\mu$ L of each sample with a concentration of 2 mg/mL and positive control of the quercetin were prepared in DMSO. Then, 3 mL of a 0.5 mg/mL solution of *β*-carotene in chloroform was pipetted into a 100 mL volumetric flask. A rotating evaporator was set to 40°C at vacuum was used to remove the chloroform. Following that, the flask's contents were shaken as 40 mg of linoleic acid, 400 mg of tween 40 to use as an emulsifier, and 100 mL of hyper oxygenated distilled water were added. The absorbance of the solutions was then immediately measured at 470 nm ( $t_0$  min) after 4.8 mL of this mixture was introduced to the prepared tubes. Subsequently, 4.8 mL of this emulsion was added to the prepared tubes, and the absorption of the solutions was immediately analyzed at 470 nm ( $t_{0min}$ ). Then the tubes were located in a thermal bath at 50°C. After 2 hours, the absorption of the tubes was measured twice ( $t_{120min}$ ). Finally, the retention percentage of *β*-carotene (or the inhibition percentage of linoleic acid) was measured using the further down formula:

$$\% \text{ retention of } \beta\text{-carotene} = \frac{\text{Absorbance}_{t_{120min}}}{\text{Absorbance}_{t_0min}}$$

#### Assay for total phenolic content (TPC)

The Folin-ciocalteu (FC) test was employed to measure the TPC of *R. lanata* (chloroform, ethyl acetate, *n*-butanol and aqueous) extracts. First, different concentrations of standard gallic acid (range 0 to 2000  $\mu$ g/mL) and extracts (5 mg/mL) were prepared and centrifuged. Then, 20  $\mu$ L of each sample was poured into separate tubes, and 1.58 mL of distilled water and 100  $\mu$ L of FC reagent were increased to the mixture and placed at room temperature for 5 minutes. Subsequently, a volume of 300  $\mu$ L of a sodium carbonate solution with a concentration of 0.18 M was introduced into the amalgamation. After 2 hours, the absorbance was analyzed at 765 nm. The absorption-concentration diagram was drawn for the gallic acid standard, and TPC was calculated as mg of gallic acid equivalent (GAE) per g of dry extract (mg GAE/g E).<sup>42</sup>

#### Assay for total flavonoid content (TFC)

The TFC of *R. lanata* extracts was determined using the aluminum chloride reagent. Different concentrations of quercetin standard (range 0-1000  $\mu$ g/mL) and (chloroform, ethyl acetate, *n*-butanol and aqueous) extracts (5 mg/mL) were prepared and centrifuged. 100  $\mu$ L of each sample were mixed with 200  $\mu$ L of distilled water. Subsequently, a volume of 40  $\mu$ L of a 10% AlCl<sub>3</sub> solution was introduced, followed by the addition of 40  $\mu$ L of a 1 M potassium acetate solution and 1120  $\mu$ L of distilled water. The resulting solution was violently agitated. The absorbance of the samples was determined at a wavelength of 415 nm after a duration of 40 minutes of incubation time, in conjunction with a blank. The quercetin standard calibration curve was drawn. TFC was calculated in terms of quercetin mg equivalent (QE) per gram of dry extract (mg/g E).<sup>43</sup> The antioxidants, TFC and TPC figures were provided in the Supplementary Data (Figures S1-S5).

#### Evaluation of antimicrobial activity

##### Test microorganisms

In this study, a selection of gram-positive bacteria including *Enterococcus faecalis* (ATCC 29737), *Bacillus pumulis* (PTCC 1274), *Staphylococcus epidermidis* (ATCC 12228), and *Staphylococcus aureus* (ATCC 25923), as well as gram-negative bacteria such as *Bacillus cereus* (PTCC 1247), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 85327), and *Escherichia coli* (ATCC 25922), and a fungus known as *Candida albicans* (ATCC 10231), were employed to assess the antimicrobial properties of *R. lanata* extracts.

##### Disk diffusion assay

Extracts of the *R. lanata*'s aerial parts (*n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous) were evaluated for antibacterial capabilities using the disk diffusion technique. First, the studied microorganisms were cultured on Mueller-Hinton agar medium (0.5 McFarland standard). Extracts were provided at a concentration (50 mg/mL) in 10% DMSO. Previously, autoclaving was performed on all paper disk, and then 30  $\mu$ L of the extract solution was transported onto the disks. Disks containing 10% DMSO were used as the negative control. Following a 37°C incubation for 24 hours, the diameter of the inhibition zone (DIZ) was reported as mean $\pm$ SD in triplicates.<sup>44</sup>

##### Determination of minimum inhibitory concentration (MIC)

In order to determination of the MIC of bacterial growth by *R. lanata* plant extracts was executed in accordance with the standards provided by the Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>45</sup> The dilution method was used in a 96-well plate. A series of dilutions from the extracts in MHB from 0.39-25 mg/mL resulting in a volume of 50  $\mu$ L were prepared from the stock mixture (50 mg/mL in DMSO 10%). A suspension of bacterial culture was prepared with a turbidity of half McFarland. This suspension was diluted 1:100 with Mueller-Hinton

broth, and then 50  $\mu\text{L}$  of it was added to each well. After incubating at 37°C for 24 hours, the wells were checked for turbidity and the growth pattern of each bacterium, and the MICs were determined based on mg/mL.<sup>46</sup>

#### Determination of the minimum bactericidal concentration (MBC)

In order to determine the MBCs, samples were taken from all the wells in which a lack of bacterial growth was observed and cultured using the surface method. For this purpose, 100  $\mu\text{L}$  of the wells that showed a lack of bacterial growth were poured on MHA culture medium, and after incubating for 24 hours, the growth of microorganisms on culture plates was monitored. The MBC of the extracts were determined by finding the lowest concentration at which bacteria did not proliferate.<sup>47</sup>

#### Assays for allelopathic potential of *R. lanata* extracts

The phytotoxicity of *R. lanata* extracts (n-hexane, chloroform, aqueous, n-butanol and ethyl acetate) was investigated against *C. campestris* seeds. Concentrations of 0.25, 0.5, 1 and 2 mg/mL from each of the extracts were provided in DMSO-water solvent (5% v/v). Separately, extracts were carefully positioned onto the sterilized filter paper, which had a diameter of 10 cm, inside the confines of the petri dish. Subsequently, a total of 25 seeds of *C. campestris* were strategically positioned on filter paper at suitable intervals. Previously, to activate and clean the seeds from microbial and fungal contamination, they were treated for 20 minutes in 96% sulfuric acid and soaked in distilled water.<sup>48</sup> The herbicide trifluralin (0.1% v/v) and DMSO diluted in water (5% v/v) were served as the positive and negative controls, respectively. The treatments were arranged in three replications. The petri dishes were kept in the darkness at 24°C until germination was carried out. During 10 consecutive days and at 24-hour intervals, germinated seeds were counted in each petri dish. Subsequently, On day 10, we measured the length and weight of the sprouting seeds.<sup>49</sup> The percentage of seed germination, seedling length and seedling weight growth inhibition for each concentration were calculated from the following equation:

$$\text{Inhibition (\%)} = \frac{S_G - S_T}{S_G} \times 100$$

Where  $S_G$  is the mean number of germinated seeds (or seedling length growth or seedling weight growth) in the negative control plate, and  $S_T$  is the sum of sprouted seeds (or seedling length growth or seedling weight growth) in the extract treatment plates. After plotting the percent inhibition-concentration graph for each extract,  $\text{IC}_{50}$  values (concentration representing half-maximal inhibition) were measured for the prevention of germination, seedling length and seedling weight cultivation.<sup>49</sup>

#### In silico molecular dynamics simulation

The Glide Schrodinger 2015-2 package was used to investigate the ligand-receptor interaction of the identified compounds from n-hexane and 70% ethanol extracts of *R. lanata*. Glutamine synthetase (GS, PDB ID: 5KHA),<sup>50</sup> 4-hydroxyphenyl pyruvate dioxygenase (HPPD, PDB ID: 3E9Y), and acetohydroxyacid synthase (AHAS, PDB ID: 3E9Y).<sup>51,52</sup> All had their protein structures were prepared on Maestro 10.2. Water molecules with less than 3 hydrogen bonds with other atoms and a distance of 5 Å were removed from the side domains in crystal structures. The pH was set in the range of 3.0 to 7.0 (biological pH). The structure of proteins was reviewed and modified in terms of having heavy metals. Disulfide bonds were created if there were any, then side chains and lost loops were restored.

The assignment of partial atomic charges was performed with the optimized potential liquid simulation (OPLS3) force field in 0.30 Å. The grid generation application was used to produce grid boxes, and their validation was completed with standard native ligands in the crystal structures of proteins.<sup>53</sup> Briefly, the process of validating the grid box was divided into two parts. The first part involved choosing the crystal structures of enzymes that contained standard native ligands. In the current instance, the enzymes' grid boxes were clear enough to fit the entire structure of the ligands. The second part involved making sure that these grid boxes were correct by considering the entire enzyme structure as a grid box. After re-docking the native standard ligands again, it was demonstrated that the grid boxes chosen in the first part were the most stable in terms of docking score. Active site box dimensions were 15 × 15 × 15 Å for GS, HPPD, and AHAS, respectively, with the following grid box coordinates: x = 78.41, y = 32.33, z = 38.16; x = -17.45, y = -24.59, z = 5.63; and x = 65.55, y = 54.99, z = 41.75.<sup>54</sup> Subsequently, ChemDraw v15 and the Chem3D module were used to draw the 2D and 3D ligand structures, respectively. The Ligand Preparation (LigPrep) module was used to prepare the tested ligands. Finally, Glide's Extra Precision (XP) mode was used to perform docking studies with the flexible ligand-receptor interaction.<sup>55</sup> Thiamine diphosphate (TDP), (2S)-3-(4-hydroxyphenyl)-2-hydroxypropionic acid (TYF) and adenosine 5'-diphosphate (ADP) were used as standard native ligands (positive controls) for the AHAS, HPPD and GS enzymes, respectively. Finally, the docking score was selected as the metric to evaluate the ligand-protein binding affinity. In other words, the level of dynamic stability of the ligand-protein system is proportional to the amount of the docking score, which is a negative number.

#### Statistical Investigation

Analysis of the data was fulfilled using the SPSS 26.0 software package. The means were compared using a one-way analysis of variance (ANOVA) accompanied by Tukey's *post-hoc*. Statistical significance was deemed for differences with p-values below 0.05.

**Table 1.** Composition of lipophilic content of *R. lanata* *n*-hexane extract.

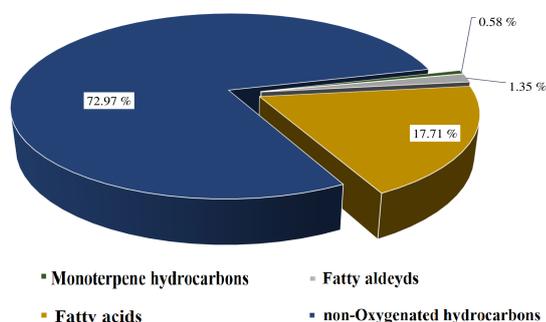
| Raw | Compounds                         | Rt <sup>a</sup> | RI STD <sup>b</sup> | EX RI <sup>c</sup> | Identification method | Percentage (%) | Lit Ref <sup>e</sup> |
|-----|-----------------------------------|-----------------|---------------------|--------------------|-----------------------|----------------|----------------------|
| 1   | Geranyl acetone                   | 18.7            | 1447                | 1453               | GC-MS <sup>d</sup>    | 0.6            | 56                   |
| 2   | Tridecanal                        | 20.5            | 1533                | 1510               | GC-MS                 | 1.3            | 56                   |
| 3   | Hexadecane                        | 22.1            | 1627                | 1600               | GC-MS                 | 0.9            | 56                   |
| 4   | Heptadecane                       | 23.1            | 1700                | 1700               | GC-MS                 | 4.9            | 56                   |
| 5   | Octadecane                        | 24.6            | 1756                | 1800               | GC-MS                 | 0.8            | 56                   |
| 6   | Palmitic acid (Hexadecanoic acid) | 27.1            | 1931                | 1965               | GC-MS                 | 13.8           | 56                   |
| 7   | <b>Nonadecane</b>                 | 27.6            | 1945                | 1900               | GC-MS                 | <b>46.8</b>    | 56                   |
| 8   | <b>Eicosane</b>                   | 29.7            | 2002                | 2000               | GC-MS                 | <b>19.5</b>    | 56                   |
| 9   | Oleic acid (9-Octadecenoic acid)  | 32.2            | 2073                | 2116               | GC-MS                 | 3.9            | 56                   |
|     | Total (%)                         |                 |                     |                    |                       | 92.6           |                      |

<sup>a</sup>Rt: Retention time<sup>b</sup>RI STD: Standard retention index from the literature.<sup>c</sup>EX RI: Standards of *n*-alkanes (C<sub>8</sub>-C<sub>21</sub>) were applied on a capillary column comprised of methylphenylsiloxane (DB-1) and the retention index was determined.<sup>d</sup>GC-MS: Gas chromatography mass spectrometry-based identification. Trace ≤0.05%.<sup>e</sup>Lit Ref: literature reference.

## Results and Discussion

### Chemical constituents of the *n*-hexane extract

In the present study, *n*-hexane extract was analyzed, targeting the identification of lipophilic compounds using GC-MS. Table 1 presents the phytochemicals that have been discovered, together with their respective percentages and RI.

**Figure 2.** Percentage of various portions in the lipophilic content of *n*-hexane extract of *R. lanata* aerial parts.

As shown in Table 1, nonadecane (46.8%) and eicosane (19.5%) were the main phytochemical constituents in the lipophilic fraction of the *n*-hexane extract from *R. lanata*. In addition, the presence of palmitic acid and oleic acid, with 13.8 and 3.9%, respectively, was proven in the lipophilic fraction. Mainly, these compounds and most of the (sub)-constituents in the lipophilic extract belong to the family of fatty acids and non-oxygenated hydrocarbons (Figure 2). These categories are usually cataloged as non-classic essential oils (non-terpene and non-phenylpropane).<sup>57</sup> The lipophilic content of the *R. lanata* *n*-hexane extract was basically poor in terms of secondary metabolites of the classical essential oil, such as monoterpenes and sesquiterpenes, which are sometimes seen in the *n*-hexane extract, as well.<sup>58</sup>

### Inspection of TPC, TFC and antioxidant activity of *R. lanata* extracts

The antioxidant properties of ethyl acetate, aqueous, chloroform and *n*-butanol extracts of *R. lanata* were estimated using diverse antioxidant assays, including

**Table 2.** Antioxidant activity and total phenolic and flavonoid contents of *R. lanata*.

| Extracts          | Antioxidant tests               |   |  |   | Total flavonoids content as quercetin (mg QE/g E) <sup>a</sup> | Total phenolics content as gallic acid (mg GAE/g E) <sup>b</sup> |
|-------------------|---------------------------------|---|--|---|--|--|
|                   | DPPH (RC <sub>50</sub> , µg/mL) | Hydrogen peroxide scavenging (IC <sub>50</sub> , µg/mL) | Ferric reducing power (EC <sub>50</sub> , µg/mL) | β-carotene/linoleic acid bleaching power (% Inhibition) |  |  |
| Chloroform        | 1005.5±10.3                     | NA  | NA   | 7.1±1.2   | 23.7±1.7   | 34.1±2.3   |
| ethyl acetate     | 21.5.0.7                        | 174.7±4.5   | 151.2±6.7  | 56.1±1.9  | 360.3±5.6  | 1081.3±5.4   |
| <i>n</i> -butanol | 77.7±1.7                        | 632.4±7.4   | 546.8±5.4  | 36.5±2.1  | 90.8±2.7   | 597.3±3.3  |
| Aqueous           | 118.4±2.0                       | 961.8±9.3   | 831.6±3.8  | 24.2±9  | 8.0±1.3  | 20.3±2.3   |
| <b>Standards</b>  |                                 |   |  |   |  |  |
| Quercetin         | 1.83±0.2                        | NT  | 13.4±3.1   | 96.1±1.3  | NT   | NT   |
| Ascorbic acid     | NT                              | 61.6±3.6  | NT   | NT  | NT   | NT   |

DPPH radical reduction method, hydrogen peroxide inhibition, ferric reducing power and  $\beta$ -carotene/linoleic acid oxidation power assays. In addition, the TPC and TFC of extracts were measured based on the equivalents to the standard of gallic acid and quercetin, respectively (Table 2).

Among the *R. lanata* extracts, ethyl acetate showed a significant antioxidant effect for all the tests performed, and the values of the factors expressing its antioxidant ability in the DPPH radical reducing, radical inhibition of  $H_2O_2$ , ferric reducing power and  $\beta$ -carotene/linoleic acid bleaching power tests were  $21.5 \pm 0.7 \mu\text{g/mL}$ ,  $174.7 \pm 4.5 \mu\text{g/mL}$ ,  $151.2 \pm 6.7 \mu\text{g/mL}$  and  $56.1 \pm 1.9 \%$ , respectively (Table 2). This extract also showed the highest TPC and TFC with values of  $1081.3 \pm 5.4 \text{ mg GAE/g E}$  and  $360.3 \pm 5.6 \text{ mg QE/g E}$ , respectively. Also, the lowest antioxidant effect in DPPH radical scavenging and  $\beta$ -carotene chelating oxygen-free radicals' assays with values of  $10.3 \pm 10.3 \mu\text{g/mL}$  ( $RC_{50}$ ) and  $7.1 \pm 1.2 \%$  inhibition) was correlated to the chloroform extract of the plant, while the minimum of TPC and TFC with values of  $20.3 \pm 2.3 \text{ mg GAE/g E}$  and  $8.0 \pm 1.3 \text{ mg QE/g E}$  was related to the aqueous fraction of the 70% ethanol extract. Approximately, similar results were obtained in a study performed by Graikou *et al.*,<sup>59</sup> the inhibition percentages of DPPH and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) reagent reduction of the *R. graeca* aerial parts methanolic extract at  $50 \mu\text{g/mL}$  concentration were equal to 24.2 and 52.2, respectively. In addition, TPC and TFC values for this extract were reported at  $66.5 \pm 1.6 \text{ mg GAE/g E}$  and  $9.7 \pm 0.1 \text{ mg QE/g E}$ , respectively. Additionally, in another study, it was demonstrated that the highest amounts of TPC and TFC of *R. umbellata*, with values of  $32.37 \text{ mg GAE/g E}$  and  $22.87 \text{ mg Rutin/g E}$ , respectively, were related to the leaf's methanol extract compared to the organs of flowers, roots and stems.<sup>60</sup>

In confirmation of the results acquired in the current study, according to the literature, the antioxidant effect of polar extracts (methanolic and ethanolic) revealed a direct relationship with TPC and TFC; in other words, the considerable oxidant inhibition of an extract is, the highest its phenolic and flavonoid content.<sup>61</sup>

Besides, the implications of the outcomes in the analysis of the antioxidant activity of fractionated polar extracts by the liquid-liquid extraction method are that by increasing the fractionation solvent's polarity (from ethyl acetate to aqueous), the amounts of TPC and TFC were reduced. In a study conducted in 2017 on the *n*-butanol, ethyl acetate, aqueous and *n*-hexane fractions obtained from liquid-liquid partitioning chromatography of the *Clinacanthus nutans* methanol extract, it was shown that the ethyl acetate portion had the significant antioxidant effect in the DPPH and ferric reducing antioxidant tests as well as the highest TPC and TFC, while the aqueous fraction had the lowest values of the mentioned tests.<sup>33</sup>

### Antimicrobial activity of *R. lanata* extracts

Nine microorganisms were used in the determination of the antimicrobial activity of the aerial parts of *R. lanata* extracts. Table 3 shows the rates of MBC, DIZ and MIC obtained for antimicrobial activity. *P. aeruginosa* and *C. albicans* were the only microorganisms that showed resistance to the extracts. Sensitive bacterial strains had DIZ and MIC values between 7-20 mm and 1.56-25 mg/mL, respectively, when exposed to the extracts. The MBC was 25 mg/mL in only two bacteria, *S. aureus* and *S. epidermidis*, which were related to chloroform, ethyl acetate and *n*-butanol extracts. The aqueous partition was inactive against all microorganisms. The highest value of DIZ was correlated to the *R. lanata* ethyl acetate extract against the gram-positive bacteria *S. epidermidis*, with a value of  $20.3 \pm 2.9 \text{ mm}$ . However, the DIZ values of all extracts were lower than those of standard disks (nystatin, tetracycline and ampicillin). Consequently, ethyl acetate extract indicated the lowest range of MIC (1.56–6.25 mg/mL) among the extracts. As mentioned in the previous section, the ethyl acetate extract had the highest TPC and TFC. Since phenolics and flavonoids show considerable activity against a broad variety of pathogenic microorganisms because of their structural characteristics, So as, with the increase in antibiotic-resistant bacterial strains, the mentioned compounds are of great importance due to their potential to replace antibiotics.<sup>62</sup> Published scientific studies have indicated that phenolic constituents such as tannic acid, rutin and epigallocatechin gallate have shown appropriate antimicrobial activity against *S. epidermidis* and *P. aeruginosa*. Most likely, the allowable antimicrobial effect of *R. lanata* ethyl acetate extract might be due to the presence of high levels of flavonoid and phenolic compounds,<sup>63</sup> some of which have been isolated and identified in our study. The most important antibacterial mechanisms of phenolic and flavonoid compounds include: disruption of nucleic acid synthesis, damage to the bacterial membrane by troubling the execution of the cytoplasmic membrane and blocking cell membrane purine, inhibition of energy-producing metabolic pathways, suppression of the attachment and formation of bacterial biofilms, alteration of the permeability of the membrane, and debilitation of the pathogenicity.<sup>62</sup>

According to the data in Table 3, the studied extracts demonstrated stronger antimicrobial capacity against gram-positive than gram-negative bacteria, which could be due to the impermeable cell wall of lipopolysaccharide present in the gram-negative bacteria.<sup>64</sup> Among the few biological studies conducted on the genus *Rindera*, the antimicrobial activity of *R. lanata* var. *canescens* aerial part essential oils (extracted from HD and MW assays) were investigated against seven selected microorganisms. The results demonstrated that the essential oils were effective only on *C. albicans* (although the results were less effective than the fluconazole standard), and the HD method's essential oil was more powerful.<sup>20</sup> Different extracts and bioactive metabolites of *R. graeca* were evaluated in vitro

**Table 3.** The DIZ, MIC and MBC values of *R. lanata* extracts in antimicrobial test.

| Microorganism<br>(Gram)   | R. lanata extracts <sup>d</sup> |                  |                  |            |      |     |               |      |     |           |      |     | Standards <sup>e</sup>       |                            |                          |
|---------------------------|---------------------------------|------------------|------------------|------------|------|-----|---------------|------|-----|-----------|------|-----|------------------------------|----------------------------|--------------------------|
|                           | n-hexane                        |                  |                  | chloroform |      |     | ethyl acetate |      |     | n-butanol |      |     | Tetracycline<br>(30 µg/disk) | Ampicillin<br>(10 µg/disk) | Nystatin<br>(30 µg/disk) |
|                           | DIZ <sup>a</sup>                | MIC <sup>b</sup> | MBC <sup>c</sup> | DIZ        | MIC  | MBC | DIZ           | MIC  | MBC | DIZ       | MIC  | MBC | DIZ                          | DIZ                        | DIZ                      |
| <i>B. pumulis</i> (+)     | 7.7±2.7                         | 25               | *                | 8.7±1.6    | 12.5 | *   | 11.7±0.7      | 6.25 | *   | 8.7±1.6   | 12.5 | *   | 27.0±0.9                     | *                          | *                        |
| <i>E. faecalis</i> (+)    | 8.7±1.2                         | 12.5             | *                | *          | *    | *   | 11.3±0.7      | 6.25 | *   | 9.7±2.3   | 12.5 | *   | 21.3±0.9                     | *                          | *                        |
| <i>S. aureus</i> (+)      | 10.7±2.3                        | 6.25             | *                | 13.7±0.1   | 6.25 | 25  | 13.7±1.0      | 1.56 | 25  | 10.7±1.4  | 6.25 | *   | 26.0±2.3                     | *                          | *                        |
| <i>S. epidermidis</i> (+) | 10.6±0.6                        | 6.25             | *                | 14.7±1.4   | 1.56 | 25  | 20.3±2.9      | 1.56 | 25  | 14.7±1.4  | 1.56 | 25  | 40.3±2.1                     | *                          | *                        |
| <i>E. coli</i> (-)        | 8.7±0.4                         | 12.5             | *                | 9.0±2.4    | 25   |     | 12.7±1.4      | 6.25 | *   | 10.7±0.8  | 12.5 | *   | *                            | 20.3±4.5                   | *                        |
| <i>K. pneumoniae</i> (-)  | 8.0±0.0                         | 12.5             | *                | 9.0±0.5    | 25   |     | 13.0±1.2      | 3.12 | *   | 9.0±3.1   | 12.5 | *   | *                            | 22.6±3.1                   | *                        |
| <i>P. aeruginosa</i> (-)  | *                               | *                | *                | *          | *    | *   | *             | *    | *   | *         | *    | *   | *                            | 16.6±2.2                   | *                        |
| <i>B. cereus</i> (-)      | *                               | *                | *                | 8.7±2.6    | 12.5 |     | 10.7±1.4      | 6.25 | *   | 9.7±1.6   | 12.5 | *   | *                            | 22.3±1.2                   | *                        |
| <i>C. albicans</i> (f)    | *                               | *                | *                | *          | *    | *   | *             | *    | *   | *         | *    | *   | *                            | *                          | 18.3±1.2                 |

<sup>a</sup> DIZ: values of the diameter of inhibition zones (mm) including of a standard disk value (6 mm).

<sup>b</sup> MIC The mentioned data reflects the minimal inhibitory concentration, values as mg/mL.

<sup>c</sup> MBC: The mentioned data reflects the minimum bactericidal concentration, values as mg/mL.

<sup>d</sup> Aqueous extract was inactive against all microorganisms.

<sup>e</sup> Mildly active (7-14 mm), very active (>14 mm), and inactive (\*).

against microorganisms. With this research, the antimicrobial effect of *n*-hexane extract of hairy roots, methanol extract of in vitro shoots, and methanol extract of aerial parts was investigated against 9 microorganisms. *S. epidermidis* and *S. aureus* showed the highest sensitivity to extracts with MIC ranges of 0.68-0.88 and 0.50-0.76 µg/mL, respectively.<sup>21</sup> Due to the limited antimicrobial studies on the genus *Rindera*, in the following, we will discuss the antimicrobial results related to similar genera in the Boraginaceae family. Hernandez *et al.*,<sup>65</sup> investigated the antimicrobial activity of hexane, chloroform and methanolic extracts of the aerial parts of *Cordia curassavica* (Jacq) against 13 bacterial strains and 5 fungal strains. The range of MICs obtained by the broth dilution method for the studied extracts was 125–2000 µg/mL. In addition to *n*-hexane, chloroform and methanolic extracts indicated the strongest effect against *Vibrio cholerae*, *Fusarium moniliforme* and *S. aureus* microorganisms, with MIC values of 125, 430 and 500 µg/mL, respectively.

Furthermore, 70% ethanol extracts of *Onosma gmelinii* demonstrated a high potential to inhibit gram-positive bacteria *S. epidermidis* and *S. aureus* with MBC of 18.3 and 73.3

µg/mL, respectively (compared to ampicillin reference with MBC of 7.8 and 3.9 µg/mL respectively).<sup>66</sup>

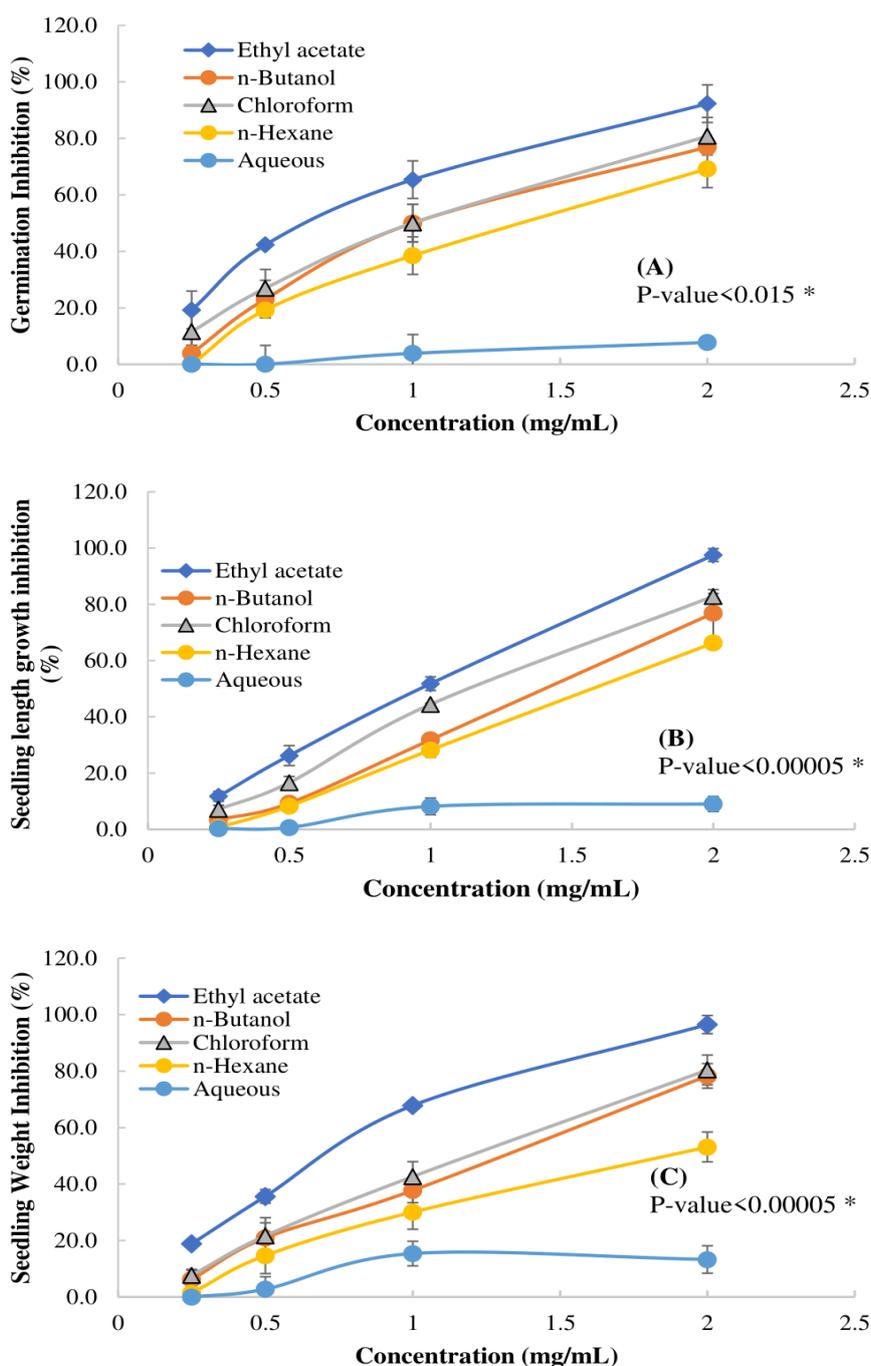
In another study, the ethanolic extract of the root of *Trichodesma indicum* (Linn.) by inhibiting the growth of microorganisms *S. aureus*, *C. albicans* and *B. subtilis* with MIC values of 19.2 µg/ml showed a satisfactory antimicrobial effect compared to the standard antibiotic (MIC = 2.4 µg/mL).<sup>67</sup> The results of the reported studies indicated a stronger effect of polar extracts on gram-positive bacteria compared to gram-negative bacteria, which is in line with the results of the present study.

#### Phytotoxicity of the *R. lanata* extracts against *C. campestris* seeds

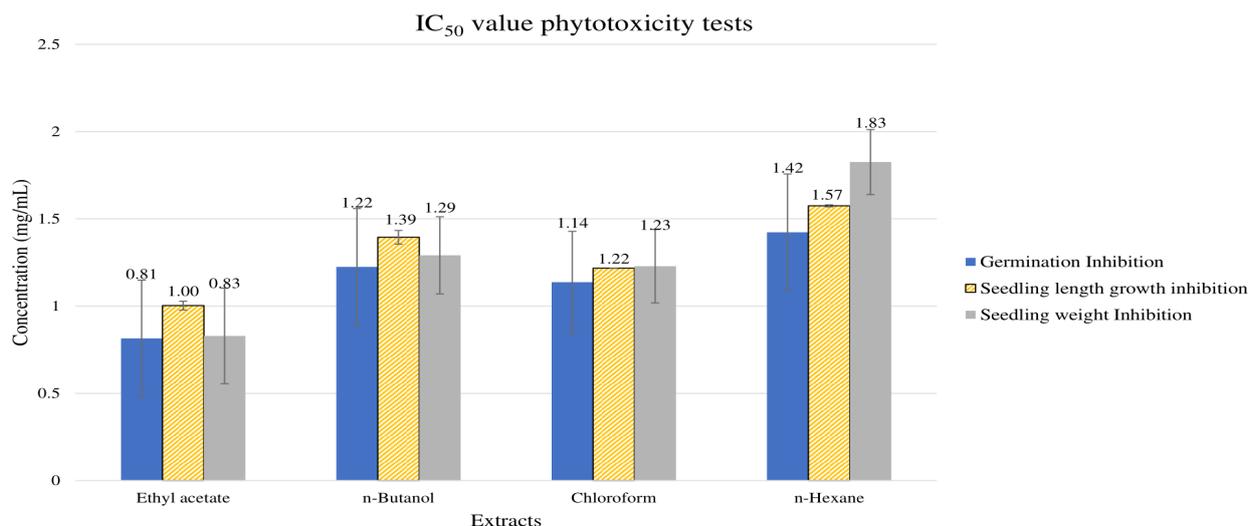
The results obtained from the phytotoxic investigation, which included the measurement of the inhibition percentage of number of germinations, seedling length, and seedling weight growth of *C. campestris* seeds, are shown in Figure 3. Based on the findings, all the extracts (except for the aqueous part) revealed a concentration-dependent increase (p-value≤0.05) in the inhibitory effect on seeds growth. The maximum average number

of germinated seeds was related to *n*-hexane, aqueous extract and the negative control group (DMSO-water (5%, v/v)), with a value of  $8.33 \pm 0.58$  out of 25 seeds of each group. In addition, the highest percentage of inhibition of seed germination was for the 2 mg/mL ethyl acetate extract, with a value of  $92.31 \pm 6.65\%$ . The highest seedling growth length after the negative group was associated to the dosage of 0.25 mg/mL aqueous and *n*-hexane extracts

with a value of 12 cm, and the lowest value was related to the ethyl acetate extract (2 mg/mL) with a rate of 0.3 cm. Additionally, according to Figure 3, the maximum and minimum percentages of inhibition of seedling length growth were observed for ethyl acetate (97.52±2.92%) and aqueous portion (0.25±0.027%) in concentrations of 2 mg/mL and 0.25 mg/mL, respectively. The average minimum and maximum seedling weights were related to the dosage



**Figure 3.** Allelopathy activity of *R. lanata* extracts against (A) germination inhibition, (B) inhibition of seedling length growth, (C) inhibition of seedling weight growth of *C. campestris*. (n=3) The standard deviation was shown as bars (\* P ≤ 0.05).



**Figure 4.** Calculated IC<sub>50</sub>s for different *R. lanata* aerial parts extracts in phytotoxicity tests against *C. campestris* seed germination (n=3). The IC<sub>50</sub> value of aqueous extract could not be reliably measured.

of 2 mg/mL of ethyl acetate extract and the concentration of 0.25 mg/mL of aqueous extract, with values of  $1.53 \pm 1.67$  and  $47.67 \pm 3.21$  mg, respectively. Interestingly, the percentage of seedling weight growth inhibition in the ethyl acetate extract with a concentration of 0.25 mg/mL ( $18.88 \pm 1.21\%$ ) was higher than that in the 2 mg/mL aqueous extract ( $4.84 \pm 13.29\%$ ), which showed that the germ inhibition activity of this extract was much greater than that of aqueous extract.

According to Figure 4, the ethyl acetate extract of *R. lanata* had the highest inhibitory effect among the extracts with IC<sub>50</sub> rates of  $0.81 \pm 0.33$ ,  $1.00 \pm 0.025$  and  $0.83 \pm 0.27$  mg/mL for The suppression of sprouting, seedling length, and seedling weight of *C. campestris* seeds, respectively, compared to the positive control, trifluralin, with IC<sub>50</sub> values equal to 0.054, 0.0415 and 0.0603 mg/mL, showing weaker inhibitory effects on sprouting, seedling length and seedling weight growth, respectively. All the extracts, except for the aqueous extract, showed a significant difference in all concentrations for inhibiting germination, seedling length, and seedling weight ( $P < 0.05$ ).

Based on the study organized by Hassannejad *et al.*,<sup>68</sup> aqueous extracts of *Salvia officinalis* L., *Melisa officinalis*, *Thymus vulgaris* L., *Rosmarinus officinalis* L. and especially *Lavandula vera* DC shown a considerable allelopathic effect in inhibiting the growth of *C. campestris* seeds. aqueous extract of *L. vera* (5% g powder per 100 mL) inhibited germination and seedling weight growth by 80% and 85%, respectively.

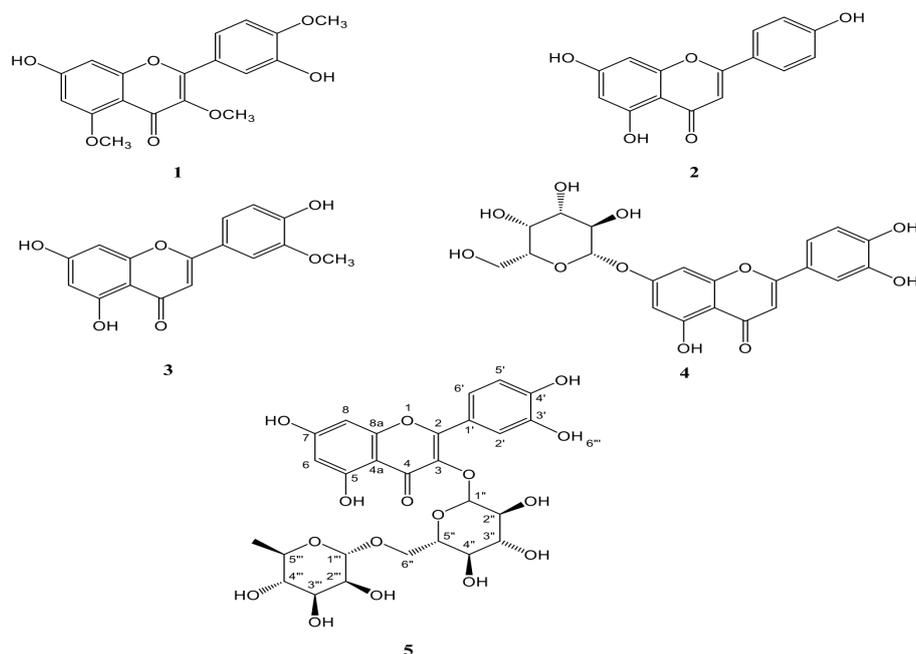
In another study, hydroalcoholic extracts of *Carum carvi* seeds with different concentrations were sprayed on *C. campestris* weed for nine days (once a day). The outcomes of the study exhibited that the concentration of 10% (w/v of water) of extract killed 66.02% of *C. campestris*.<sup>69</sup> As reported by the literature, monoterpenes<sup>70</sup> and fatty acids such as palmitic acid, linoleic acid and oleic acid<sup>71</sup> were characterized as allelochemicals against weeds.

Due to the presence of some of these compounds, such as geranyl acetone (0.58%), palmitic acid (13.77%) and oleic acid (3.95%) in the *n*-hexane extract of *R. lanata*, the germination inhibition effect of this extract could be attributed to these compounds. Phenolic compounds are a large category of allelochemicals. Polyphenol benzoic and cinnamic derivatives, depsides, flavonoids, depsidones and other aromatic chemicals are all examples of such structures with varying degrees of chemical complexity.<sup>72</sup> Phenolic compounds and especially flavonoids in plants play a critical character in auxin transport, root and stem growth, pollination, the quenching of free oxygen radicals, and the signaling of symbiotic microorganisms. In addition, some of them have considerable antibacterial, anti-fungal and anticancer effects.<sup>73,74</sup> These chemicals are transported into the rhizosphere of soil by the roots, and this release occurs either as a direct result of root secretion or as a result of the destruction of plant tissue over time and causes the induction of autotoxicity and allelopathy by the host plant.<sup>75</sup> Following the isolated flavonoid and the high values of TPC and TFC in ethyl acetate and *n*-butanol extracts of *R. lanata* aerial parts, the observed phytotoxic effect might be caused by these compounds.

Allelochemicals with a phenolic structure have a high affinity for a wide range of plant enzymes and proteins, for example: interaction with indoleacetic acid (IAA) oxidase and changing the level of phytohormone IAA,<sup>76,77</sup> inhibition of catalase, maltase, nicotinamide adenine dinucleotide (NADH) oxidase enzymes,<sup>78</sup> invertase and phosphatase, which leads to increased oxidative stress and induction of cell death in plants.<sup>79</sup> For this reason, we decided to use molecular docking interactions to find the relationship between *R. lanata* compounds and the relative allelopathic activity.

#### Identification of the isolated substances

The chemical structures of the isolated compounds are



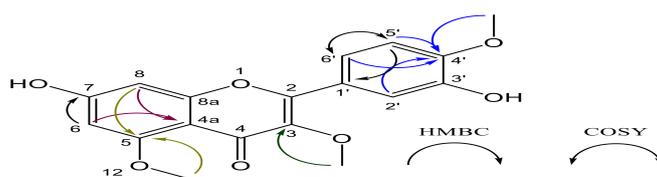
**Figure 5.** Chemical structures of compounds 1-5 isolated from *R. lanata* aerial parts.

shown in Figure 5, including 3, 5, 4'-tri-*o*-methoxyquercetin (1), apigenin (2), chrysoeriol (3), luteolin-7-*o*-glucoside (4) and rutin (5) that were identified by 1D and 2D NMR spectroscopy. Compound (1) was a flavonoid whose structure was not previously reported in the literature. Additionally, chrysoeriol (3)<sup>80</sup> and luteolin-7-*O*-glucoside (4)<sup>81</sup> were isolated and reported for the first time in this plant. Apigenin (2)<sup>82</sup> and rutin (5)<sup>83</sup> were found in the methanolic extract of the aerial parts of *R. lanata* in a study using the LC-MS/MS method.<sup>19</sup>

3, 5,4' methoxy quercetin (1) - yellow amorphous powder (7 mg); <sup>1</sup>H-NMR (DMSO, 500 MHz):  $\delta_{\text{H}}$  3.55 (3H, s, C4'-O-CH<sub>3</sub>), 3.76 (3H, s, C3-O-CH<sub>3</sub>), 3.93 (3H, s, C5-O-CH<sub>3</sub>), 6.76 (1H, s, H-6), 6.88 (1H, s, H-8), 7.10 (1H, d, 8 Hz, H-5'), 7.46 (1H, d, 2 Hz, H-2'), 7.56 (1H, dd, 8, 2Hz, H-6'). <sup>13</sup>C-NMR (DMSO, 125 MHz):  $\delta_{\text{C}}$  56.1 (C4'-O-CH<sub>3</sub>), 57.4 (C5-O-CH<sub>3</sub>), 60.2 (C3-O-CH<sub>3</sub>), 91 (C-8), 103 (C-6), 105 (C-4a), 112 (C-5'), 113 (C-2'), 118 (C-6'), 122 (C-1'), 132

(C-3), 147 (C-3'), 152 (C-4'), 153 (C-8a), 163 (C-2), 159 (C-7), 164 (C-5), 182 (C-4).

The <sup>1</sup>H-NMR spectrum of this compound (1) consisted of three methoxy groups in the  $\delta_{\text{H}}$  3.55,  $\delta_{\text{H}}$  3.76 and  $\delta_{\text{H}}$  3.93 regions, and 5 hydrogen signals in the aromatic region indicate the flavonoid structure. The doublets at  $\delta_{\text{H}}$  7.11 (d, 8.3 Hz) and doublet doublets 7.56 (dd, 8.3, 2.0 Hz) demonstrated ortho-coupled H-atoms in aromatic ring which is related to H (5') and H (6'), respectively, which are related in the COSY spectrum. Figure 6 shows the COSY and HMBC correlations in compound (1). Furthermore, two peaks at  $\delta_{\text{H}}$  6.76 and 6.88 related to H (6) and H (8) in the meta position to each other. The hydrogens of the methoxy group  $\delta_{\text{H}}$  3.55, H (2'), H (6') and H (5') showed correlation with  $\delta_{\text{C}}$  of 152 ppm in the HMBC spectrum, which revealed that the methoxy group was linked to C (4'). Moreover, the methoxy groups  $\delta_{\text{H}}$  3.93, H (8) in the HMBC spectrum were associated with  $\delta_{\text{C}}$  159, indicating the methoxy group is bonded to C (5). In addition, in



**Figure 6.** Chemical structures of compound 1 with HMBC and COSY correlations.

the HMBC spectrum of the methoxy group hydrogens,  $\delta_H$  3.76 was related to  $\delta_C$  of carbon 132, representative of the C (3) position of the flavonoid. Finally, the isolated compound's chemical structure was identified as 3, 5, 4'-tri-*o*-methylquercetin (**1**), a new compound that was purified from the ethyl acetate part of *R. lanata* aerial parts. Full spectral information on the isolated compounds is mentioned below:

Apigenin (**2**) - white powder (6 mg);  $^1\text{H-NMR}$  (DMSO, 500 MHz):  $\delta_H$  6.15 (1H, s, H-6), 6.45 (1H, s, H-8), 6.59 (2H, d, 8.5 Hz, H-3',5'), 7.81 (1H, d, 8.5 Hz, H-2',6').  $^{13}\text{C-NMR}$  (DMSO, 125 MHz): 93.9 (C-8), 98.8 (C-6), 103 (C-3), 103 (C-4a), 115 (C-5'), 127 (C-2',6'), 121 (C-1'), 157 (C-8a), 159 (C-4'), 161 (C-2), 161 (C-5), 164 (C-7), 181 (C-4).

Chrysoeriol (**3**) - yellow amorphous powder (7mg);  $^1\text{H-NMR}$  (DMSO, 500 MHz):  $\delta_H$  3.89 (3H, s, C3''-O-CH<sub>3</sub>), 6.19 (1H, d, 2.3 Hz, H-6), 6.48 (1H, d, 2Hz, H-8), 6.9 (1H, d, 9Hz, H-5''), 7.51 (2H, dd, 9, 2Hz, H-6'', 2'').  $^{13}\text{C-NMR}$  (DMSO, 125 MHz):  $\delta_C$  55.3 (C3''-O-CH<sub>3</sub>), 93.9 (C-8), 98.2 (C-6), 103.3 (C-4a), 115 (C-5''), 109.8 (C-2''), 119.9 (C-6''), 121.7 (C-1''), 102 (C-3), 147.7 (C-3''), 150.7 (C-4''), 163 (C-2), 157.8 (C-8a), 160.9 (C-5), 164 (C-7), 181 (C-4).

Luteolin-7-*O*-glucoside (**4**) - white amorphous powder (7 mg);  $^1\text{H-NMR}$  (DMSO, 500 MHz):  $\delta_H$  3.25 (1H, ,m, H-2''), 3.34 (1H, ,m, H-3''), 3.4 (1H, ,m, H-4''), 3.57 (1H, ,m, H-5''), 3.57 (1H, ,m, H-6''), 3.78 (1H, d, 11 Hz, H-6''), 5.10 (1H, d, 7.7 Hz, H-1''), 6.49 (1H, d, 2 Hz, H-6), 6.75 (1H, s, H-3), 6.83 (1H, d, 2Hz, H-8), 6.96 (1H, d, 8 Hz, H-5'), 7.46 (1H, d (2 Hz), H-2'), 7.58 (1H, dd (8.5, 2 Hz), H-6').  $^{13}\text{C-NMR}$  (DMSO, 125 MHz):  $\delta_C$  60.9 (C-6''), 69.5

(C-2''), 72.9 (C-3''), 76.7 (C-4''), 76.9 (C-5''), 94.9 (C-8), 99 (C-6), 100 (C-1''), 102.7 (C-3), 106 (C-4a), 113 (C-2''), 115 (C-5''), 119 (C-6'), 128 (C-1'), 145 (C-3'), 150 (C-4'), 157 (C-8a), 161 (C-5), 162 (C-7), 164 (C-2), 181 (C-4).

Rutin (**5**) - yellow amorphous powder (6mg);  $^1\text{H-NMR}$  (DMSO, 300 MHz):  $\delta_H$  0.97 (3H, d, 6.17Hz, C5'''-CH<sub>3</sub>), 3.04 (1H, ,m, H-3'''), 3.07 (1H, ,m, H-5'''), 3.21 (1H, ,m, H-4''), 3.21 (1H, ,m, H-5''), 3.24 (1H, ,m, H-2''), 3.27 (1H, ,m, H-4''), 3.27 (1H, ,m, H-3''), 3.37 (1H, ,m, H-2''), 3.28 (1H, ,m, H-6''), 3.71 (1H, d, 10 Hz, H-6''), 4.37 (1H, s, H-1''), 5.34 (1H, d, 7.2 Hz, H-1''), 6.18 (1H, d, 2 Hz, H-6), 6.39 (1H, d, 2 Hz, H-8), 6.84 (1H, d, 8.8 Hz, H-5'), 7.52 (1H, d, 2.14 Hz, H-2'), 7.58 (1H, dd, 8.8, 2.14 Hz, H-6').  $^{13}\text{C-NMR}$  (DMSO, 75 MHz): 17.6 (C5'''-CH<sub>3</sub>),  $\delta_C$  66.9 (C-6''), 68.5 (C-4''), 69.5 (C-3''), 70.2 (C-2''), 70.4 (C-4''), 71.7 (C-5''), 73.9 (C-4''), 75.7 (C-2''), 76.34 (C-5''), 93.7 (C-8), 98 (C-6), 100 (C-1''), 101 (C-1''), 104.5 (C-4a), 115.3 (C-5'), 116.4 (C-2'), 121 (C-6'), 121 (C-1'), 133 (C-3), 144.4 (C-3'), 148 (C-4'), 156.5 (C-2), 156.3 (C-8a), 160.9 (C-5), 164.5 (C-7), 177 (C-4). NMR spectra were provided in Supplementary Data (Figures S6-S25).

#### Molecular interactions in enzyme active sites

Ligand-receptor interactions analysis of 14 identified compounds in ethyl acetate, *n*-hexane and *n*-butanol extracts were performed to measure the affinity for AHAS, HPPD and GS enzymes through Extra Precision (XP) mode in Glide. After protein preparation, Ramachandran diagrams of each enzyme indicated a suitable empirical dispensation of the amino acid residues based on the

**Table 4.** Ligand-receptor affinity of the identified compounds from ethyl acetate, *n*-butanol and *n*-hexane extracts of *R. lanata* aerial parts on GS, AHAS and HPPD enzymes.

| No. | Name            | Formula  | Relevant extracts | Targeting proteins | Docking score (kJ/mol) | Binding sites  |
|-----|-----------------|--|-------------------|--------------------|------------------------|----------------|
| 1   | Geranyl acetone | C <sub>13</sub> H <sub>22</sub> O              | <i>n</i> -hexane  | GS                 | -4.646                 | GLN211         |
|     |                 |  |                   | AHAS               | -2.585                 | *              |
|     |                 |  |                   | HPPD               | -5.898                 | ASN423         |
| 2   | Tridecanal      | C <sub>13</sub> H <sub>26</sub> O              | <i>n</i> -hexane  | GS                 | -4.767                 | ARG311, ARG316 |
|     |                 |  |                   | AHAS               | -0.397                 | TYR276         |
|     |                 |  |                   | HPPD               | -4.973                 | ASN423         |
| 3   | Hexadecane      | C <sub>16</sub> H <sub>34</sub>                | <i>n</i> -hexane  | GS                 | -2.048                 | *              |
|     |                 |  |                   | AHAS               | -0.227                 | *              |
|     |                 |  |                   | HPPD               | -1.610                 | *              |
| 4   | Heptadecane     | C <sub>17</sub> H <sub>36</sub>                | <i>n</i> -hexane  | GS                 | -2.323                 | *              |
|     |                 |  |                   | AHAS               | -0.281                 | *              |
|     |                 |  |                   | HPPD               | -2.073                 | *              |
| 5   | Octadecane      | C <sub>18</sub> H <sub>38</sub>                | <i>n</i> -hexane  | GS                 | -2.361                 | *              |
|     |                 |  |                   | AHAS               | -0.689                 | *              |
|     |                 |  |                   | HPPD               | -2.080                 | *              |
| 6   | Palmitic acid   | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | <i>n</i> -hexane  | GS                 | -5.965                 | ARG316, ASN38  |
|     |                 |  |                   | AHAS               | -1.72                  | ARG246, ILE396 |
|     |                 |  |                   | HPPD               | <b>-5.963</b>          | PHE419, FE502  |

Table 4. Continued.

| No. | Name                               | Formula   | Relevant extracts | Targeting proteins | Docking score (kJ/mol) | Binding sites                                       |
|-----|------------------------------------|---|-------------------|--------------------|------------------------|---|
|     |                                    |   |                   | GS                 | -1.993                 | *   |
| 7   | Nonadecane                         | C <sub>19</sub> H <sub>40</sub>   | <i>n</i> -hexane  | AHAS               | -0.040                 | *   |
|     |                                    |   |                   | HPPD               | -2.478                 | *   |
|     |                                    |   |                   | GS                 | -2.407                 | *   |
| 8   | Eicosane                           | C <sub>20</sub> H <sub>42</sub>   | <i>n</i> -hexane  | AHAS               | -0.764                 | *   |
|     |                                    |   |                   | HPPD               | -2.537                 | *   |
|     |                                    |   |                   | GS                 | <b>-7.265</b>          | ARG316, ASN54, TRP53                                |
| 9   | Oleic acid                         | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>  | <i>n</i> -hexane  | AHAS               | -1.881                 | TYR276, GLY275                                      |
|     |                                    |   |                   | HPPD               | <b>-6.490</b>          | PHE419, FE502                                       |
|     |                                    |   |                   | GS                 | <b>-10.422</b>         | ARG316, TYR328, THR39, GLN201, GLU320, ARG38, LYS52 |
| 10  | Rutin (5)                          | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>   | <i>n</i> -butanol | AHAS               | <b>-10.152</b>         | SER398, HIS221, ARG216, ILE396, ASP397              |
|     |                                    |   |                   | HPPD               | <b>-11.454</b>         | GLN293, SER263, PHE424, PHE419, FE502, PRO336       |
|     |                                    |   |                   | GS                 | <b>-9.640</b>          | TYR328, TRP53, ARG316, ASN54, ARG311, MN1081        |
| 11  | Luteolin-7-O-glucoside (4)         | C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>   | <i>n</i> -butanol | AHAS               | <b>-7.386</b>          | ARG279, TYR276, ARG272, GLY275                      |
|     |                                    |   |                   | HPPD               | <b>-10.989</b>         | PHE381, PHE419, FE502, PRO336                       |
|     |                                    |   |                   | GS                 | -6.125                 | SER253, TYR328, GLN201, ARG38                       |
| 12  | Chrysoeriol (3)                    | C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>  | ethyl acetate     | AHAS               | -5.467                 | ARG279, ILE396, ARG246, LYS220                      |
|     |                                    |   |                   | HPPD               | -7.741                 | GLN293, PHE381, PRO336                              |
|     |                                    |   |                   | GS                 | <b>-7.693</b>          | SER253, TYR328, GLN201, ARG38                       |
| 13  | Apigenin (2)                       | C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>  | ethyl acetate     | AHAS               | -5.705                 | LEU183, ILE396, LYS220                              |
|     |                                    |   |                   | HPPD               | -8.128                 | GLN293, PHE381, PRO336                              |
|     |                                    |   |                   | GS                 | <b>-5.881</b>          | ARG38   |
| 14  | 3, 5, 4'-tri-O-methylquercetin (1) | C <sub>18</sub> H <sub>16</sub> O <sub>7</sub>  | ethyl acetate     | AHAS               | -3.748                 | LEU224, ARG272                                      |
|     |                                    |   |                   | HPPD               | -5.523                 | LYS220, ARG246, ILE396                              |
| 15  | ADP                                | C <sub>10</sub> H <sub>15</sub> N <sub>5</sub> O <sub>10</sub> P <sub>2</sub>               | *                 | GS                 | -10.550                | ASN54, ARG316, TYR328, SER187, SER253               |
| 16  | TDP                                | C <sub>12</sub> H <sub>19</sub> N <sub>4</sub> O <sub>7</sub> P <sub>2</sub> S <sup>+</sup> | *                 | AHAS               | -6.551                 | ARG279, SER398, ARG246, ILE396, ASP397              |
| 17  | TYF                                | C <sub>9</sub> H <sub>10</sub> O <sub>4</sub>   | *                 | HPPD               | -6.644                 | ASN423, PHE381, PHE424, PHE419, FE502               |

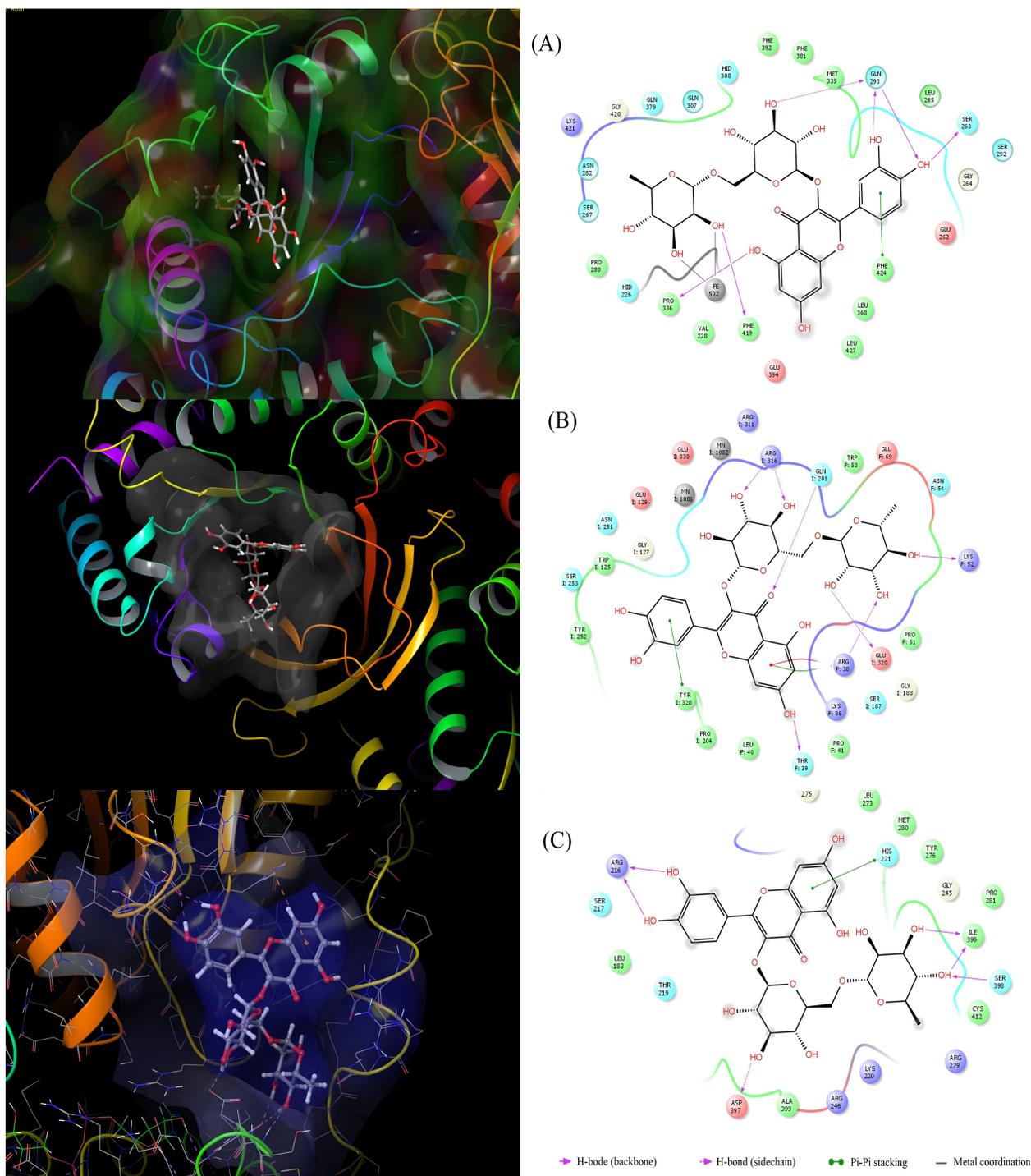
dihedral torsion angles  $\phi$  and  $\psi$ .<sup>55</sup>

Subsequently, the affinity of ligands for GS, AHAS and HPPD enzymes with the docking score parameter was compared with the native standard ligands ADP, TDP and TYF, respectively (Table 4). Among the investigated compounds, rutin had the highest interaction with the GS, AHAS and HPPD enzymes, with docking scores of -10.422, -10.152 and -11.454 kJ/mol, respectively. Basically, the presence of this compound inhibited the enzymes.

Investigation of the major interactions between rutin and the active site of HPPD protein exhibited that there were six H-bonding connections of the OH groups from the molecule with GLN293, SER263, PHE419 and PRO336 amino acids, and  $\pi$ - $\pi$  stacking between PHE424 and aromatic rings, as well as metal coordination of OH groups in sugars with FE502 that created a strong bond between the ligand and the HPPD receptor (Figure 7). The interactions of rutin with the binding pockets of the HPPD, AHAS

and GS are shown in Figure 7. In general, the presence of OH groups and unsaturated carbonyl or alkene bonds in the structure of ligands increases the number of H bond correlation of OH groups and pi interaction of aromatic ring in the active site and increased the docking score.<sup>84</sup> This reason for luteolin-7-*O*-glucoside also showed a good interaction with GS, AHAS and HPPD enzymes (Table 4). There have been various kinds of investigations on molecular docking between phenolic compounds and GS,

AHAS and HPPD enzymes; for example, quinones have a high inhibitory effect on HPPD.<sup>85</sup> Additionally, quercetin-3-*O*- $\beta$ -D-glucoside flavonoid indicated a high affinity for the GS enzyme isolated from *Mycobacterium* spp.<sup>86</sup> Also, a high inhibitory effect of oxygenated compounds in essential oils on GS, AHAS and HPPD enzymes was exhibited *in vitro* and *in vivo*.<sup>87</sup> In addition, many herbicides have a phenolic domain in their structure, that could be considered as their main pharmacophore.<sup>88</sup>



**Figure 7.** 2D and 3D ligand-receptor interactions of the rutin isolated from *n*-butanol extracts of *R. lanata* aerial parts on GS, AHAS and HPPD enzymes. (A) HPPD enzyme, (B) GS enzyme, (C) AHAS enzyme active sites.

In general, dynamic inhibition or energetic stability of HPPD, GS and AHAS enzymes by fatty acids, monoterpenes, phenolics and flavonoids of *R. lanata* aerial parts extracts was manifested by establishing H bonds and  $\pi$ - $\pi$  stacking in the active sites, could cause the phytotoxic activity of *R. lanata* upon the microorganisms around the plant.

### Future directions

While this study has provided valuable insights into the molecular interactions of *R. lanata* compounds with key enzymes involved in the phytotoxic process and the biological properties of its extracts, opportunities for further research have emerged. One area of future exploration is the *in vivo* validation of the plant's biological inhibitory effects. Conducting animal or human studies to evaluate the effect of purified compounds on enzyme activity in a biological system can provide important insight into their potential therapeutic applications.

Following, further study is warranted to elucidate the accurate mechanism of the inhibitory effects of the identified compounds on GS, AHAS and HPPD enzymes. This can include detailed kinetic studies, structural analyzes and molecular dynamics simulations to discover the molecular interactions at play both *in vivo* and *in vitro* using specific enzymatic assay Kits.

In addition, expanding the scope of current study to explore various medical attributes of these substances, such as their anti-inflammatory and antibacterial effects on a resistant bacterial strain, can reveal more therapeutic potential of *R. lanata*.

Finally, investigating the herbicidal effects of compounds or plant extracts in the existing field environment may lead to the discovery of novel enzyme inhibitors with specific herbicidal properties. Addressing the aforementioned unanswered questions and conducting the recommended studies will help us comprehend the applicable biological effects of these chemicals and their enzyme interactions, leading to breakthroughs in medicine and agriculture.

### Limitations and constraints

Several limitations that may affect the generalizability of our findings include: First, the *in vitro* nature of the experiments performed may have different results in biological systems. Although the current report provided useful insights into the inhibitory effects of *R. lanata* compounds and extracts, additional validation via *in vivo* investigations is required to confirm their physiological significance and possible therapeutic advantages.

Additionally, the phytotoxic potential focused on a particular set of enzymes (GS, AHAS, and HPPD) and a select group of compounds from *R. lanata*. It is possible that different enzymes or plant systems react differently to these compounds, so further studies are needed to evaluate their generalizability. Furthermore, in the developed model, biological impacts based on component concentration-dependent effects were not thoroughly examined.

Understanding the dose-response relationships and possible toxicity profiles of these substances is critical for assessing their safety and efficacy in practical applications. Finally, environmental and agricultural consequences, potential effects on non-target organisms, environmental persistence and the development of resistance to the use of these bioactive compounds as enzyme inhibitors or herbicides require more consideration.

### Conclusion

Limited studies have been conducted on the molecular content and bioactivities of *R. lanata*. In current study, the phytochemical analysis of the hydroalcoholic portion of the aerial parts of *R. lanata* led to the purification of a new flavonoid and apigenin (2), chrysoeriol (3), luteolin-7-*o*-glycoside (4) and rutin (5) that were already known. Ethyl acetate extract indicated the maximum antioxidant activity in the DPPH reagent reduction, hydrogen peroxide inhibition, ferric reducing ability and  $\beta$ -carotene/linoleic acid bleaching power tests. In addition, this extract had the highest TPC and TFC values. *S. epidermidis* showed maximum sensitivity with satisfactory DIZ, MIC and MBC values against the extracts among the other assessed microorganisms. Besides, the ethyl acetate extract showed the highest phytotoxicity in inhibiting germination, seedling length growth and seedling weight growth of *C. campestris* seeds. The obtained data from the *in silico* modern molecular interaction study of the identified compounds from *n*-hexane and hydroalcoholic extract showed that rutin and luteolin-7-*O*-glycoside possessed very high affinity and docking scores on HPPD, GS, and AHAS enzymes, which were comparable with ADP, TDP and TYF. As a suggestion, the isolated molecules could be introduced as lead compounds for the development of natural herbicides. Nevertheless, to completely understand the mechanism of action of the most important bioactive phytochemicals against a broad variety of weeds, further investigations are suggested.

### Ethical Issues

The research received approval from the Vice Chancellor for Research at Tabriz University of Medical Sciences, using the code IR.TBZMED.VCR.REC.1398.146.

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### Author Contributions

Hadi Ghanbari: Investigation, methodology, Formal analysis, Funding Acquisition, Validation, Writing—Original draft, Sanaz Hamedeyazdan: Conceptualization, Methodology, Project Administration, Visualization. Reza Ghanbari: Formal Analysis, Resources. Mostafa Alilou: Resources, Project Administration. Abbas Delazar:

Resources, Data Curation. Samad Nejad Ebrahimi: Validation. Amirreza Nazemiyeh: Data Curation, Mohammad Yousef Memar: Investigation, Data Curation. Hossein Nazemiyeh: Conceptualization, Methodology, Funding acquisition, Visualization, Writing—Original draft.

### Conflict of Interest

The authors declare no conflict of interest.

### Supplementary Data

The antioxidants, TFC and TPC figures (Figure S1-S5) and NMR spectra (Figures S6-S25) can be downloaded at: <https://doi.org/10.34172/PS.2024.5>.

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