Comprehensive analysis of Moroccan middle atlas rosemary: phytochemical profiling, antioxidant activity, and molecular docking

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Abstract. This study assessed the total flavonoids, polyphenols, condensed tannins, and antioxidant activity (*in vitro* and *in silico*) of aqueous and organic extracts of fresh Moroccan rosemary (*Rosmarinus officinalis*) and hydro-distillation residues. Quantitative methods identified distinct compound profiles. Notably, the hydro-acetone composite exhibited heightened polyphenolic concentrations in both the rosemary samples. Conversely, hydro-methanolic extracts showcased elevated flavonoid levels (RD: 68.4 ± 1.1 mg EC/g MS, RF: 47.1 ± 1.0 mg EC/g MS) alongside condensed tannins (RF: 19.3 ± 0.2 mg EC/g MS, DR: 11.8 ± 0.1 mg EC/g DM). HPLC-ESI-MS analysis provided profound insights into the chemical composition of the hydromethanolic, hydroacetone, and aqueous extracts, identifying 20 compounds, predominantly phenolic diterpenes, flavonoids, and organic acids. Furthermore, rosemary extracts exhibited robust antioxidant activity. Molecular docking analyses revealed specific interactions between key rosemary compounds (Carnosol, Carnosic Acid, and Rosmarinic Acid)–and target proteins, shedding light on their potent antioxidant properties and providing valuable insights for pharmaceutical and therapeutic applications. By revealing the intricate phytochemical characterization of rosemary from the Moroccan Middle Atlas, this study enriches our understanding of its composition and paves the way for its potential applications in pharmaceuticals, supplements, and natural additives, enhancing its industrial significance.

1 Introduction

Cellular damage driven by oxidative stress and reactive oxygen species is a prominent contributor to the emergence of chronic human ailments including Crohn's disease, cardiovascular conditions, specific cancers, and neurodegenerative disorders. In the cellular stratum, oxidative stress-induced perturbations inflict profound metabolic imbalances

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including protein oxidation, lipid peroxidation, membrane disruption, and DNA impairment [1-3]. Consequently, identifying natural sources of potent antioxidants is paramount for mitigating these health risks [4].

In the context of these health challenges, Moroccan aromatic and medicinal plants have emerged as invaluable resources that offer a reservoir of bioactive compounds with potential therapeutic benefits [5]. In particular, within the Lamiaceae family, these plants harbor an array of bioactive compounds that are promising for addressing various health concerns [6]. *Rosmarinus officinalis* L., is a medicinal plant with antioxidant potential, attributed to its phytochemical components [7]. During storage and extraction of rosemary, carnosic acid is partly transformed into carnosol and other diterpenes such as rosmanol, each of which has antioxidant benefits. Rosmanol, with its enhanced antioxidant activity along with carnosol, thereby offering robust natural alternatives [8]. Compounds such as epirosmanol, isorosmanol, rosmaridiphenol, and rosmariquinone bolster the protective properties of plants [9], highlighting the potential of natural antioxidants such as rosemary and underpinning the relevance of Moroccan aromatic and medicinal plants, particularly those within the Lamiaceae family.

Rosemary is a perennial plant indigenous to the Mediterranean region. Extracts from rosemary are widely employed in culinary practices, food preservation, cosmetics, and phytotherapy due to their antimicrobial and anti-inflammatory properties, as well as their potential to prevent cardiovascular diseases and diabetes [10]. In folk medicine, rosemary extract has been traditionally used to address a range of conditions including urinary disorders, hair loss, nervous disorders, chronic weakness, and peripheral vascular diseases. It is also known for its traditional use as an astringent, tonic, rubefacient, carminative, anti-inflammatory, expectorant, antispasmodic, emmenagogue, digestive, diaphoretic, choleretic, and diuretic [11-12]. Rosemary is gaining interest owing to its antioxidant and health-promoting properties [13]. While rosemary is traditionally used in folk medicine, the scope of research on Moroccan rosemary remains relatively limited, warranting extensive investigation of its phytochemical content. Further research is imperative to validate its efficacy in treating these conditions.

This study aimed to examine the phytochemical profile of aqueous and hydrodistillation residue extracts of fresh rosemary (*Rosmarinus officinalis* L.) grown spontaneously in the Sekkoura M'daz region in the Moroccan Middle Atlas. Through rigorous qualitative and quantitative phytochemical analyses, as well as *in vitro* antioxidant activity assessment and molecular docking of rosemary's main compounds, this study aims to shed light on the complex chemical composition of rosemary.

2 Materials and methods

2.1 Plant material and study site

Plant material, comprising the wild aerial parts of *Rosmarinus officinalis*, was carefully harvested at the vibrant Skoura M'daz site. Situated in the historic Fez-Boulemane region in the central part of northern Morocco, the Skoura M'daz site boasts geographical coordinates between approximately 33° 10'-33° 30' north latitude and 4° 10-4° 50' east longitude.

2.2 Plant extract preparation

Three solvents were tested namely methanolic extract (70% (v/v)), acetonic extract (70% (v/v)), and infused extract to come close to traditional preparations. Ten grams of vegetable powder from fresh rosemary leaves were mixed with each extraction solvent. The distillation

residue was filtered to separate the aqueous distillation phase from the solid phase (leaves resulting from distillation). The filtrate was concentrated using a Rotavapor Buchi rotary evaporator, while the leaves were dried at 37°C until a constant mass was achieved. Ten grams of the recovered leaves underwent the same extraction procedure, using a combination of methanol and aqueous acetone.

The volume of the solvent must be sufficient for the matrix to remain immersed during the extraction. The optimum solid-liquid ratios, which are most often found in the literature, are generally between 1/10 and 1/50 (m/v). A ratio of 1/10 (m / v) was used. The extraction yield was calculated relative to the total weight of vegetable powder.

2.3 Extraction yield

In order to accurately determine the yield, it is essential to utilize a standardized method. In this study, the extraction yields were calculated by applying a specific formula relative to the mass of the vegetable powder [14]. Employing a standardized method allows researchers to ensure the reliability and reproducibility of their results, leading to more precise conclusions regarding the effectiveness of various extraction techniques. The extraction yields were calculated relative to the mass of the vegetable powder (ten grams) according to the following formula [14]:

$$Yield\ (\%) = (Mass\ of\ extract) / (Mass\ of\ the\ sample) \times 100 \quad (1)$$

2.4 HPLC-ESI-MS analysis conditions

Rosemary extracts were subjected to analysis using high-performance liquid chromatography coupled with negative electrospray mass spectrometry (HPLC-ESI-MS). The extracts were dissolved in a solution of methanol and water at a ratio of 50:50 (v/v), resulting in a concentration of 800 µg/mL. To ensure sample integrity, the solutions were filtered through a 0.25-µm filter and then stored at -80°C. This storage condition was chosen to prevent potential degradation before the commencement of analysis.

The analytical process employed a Dionex UHPLC (Ultimate 3000) system with a standard automatic thermostat-controlled sampler. A specialized HPLC column, ACE C18 (4.6 mm × 150 mm, 3 µm), was used for chromatographic separation. For each injection within the HPLC system, 10 µL of the sample was introduced and the autosampler temperature was maintained at 4°C to mitigate any risks of thermal degradation.

The HPLC system was seamlessly coupled with a Thermo Fisher Scientific Mass Spectrometer (TSQ Endura) via an ESI interface capable of operating in both positive and negative modes. The detection range spanned from 50 to 1100 m/z, with nitrogen serving as both mist and drying gas. ESI parameters were optimized as follows: spray voltage set at 4000V for positive mode and 2900V for negative mode; capillary temperature and vaporizer temperature both set at 150ºC; sheath gas at 20arb, Aux Gas at 11arb, and Sweep gas at 4arb; Q1 and Q3 (FWHM) at 0.7. The collision energy values for MS/MS experiments were carefully tailored: m/z 100 at 20 eV, m/z 500 at 35 eV, and m/z 1000 at 50 eV.

The identification of key compounds relied on a comprehensive approach involving the interpretation of mass spectra of the detected molecules. This interpretation was complemented by information obtained from existing literature and databases.

2.5 Total polyphenol

Total polyphenol content was determined using the following procedure: 1 ml of Folin-Ciocalteu reagent – ten times the standard concentration, was introduced to 200 µl of either

the sample or standard, which had been duly prepared in methanol through appropriate dilutions [15]. Following a 4-minute interval, 800 µl of sodium carbonate solution at a concentration of 75 mg/ml was added to the reaction medium. The mixture was then incubated for 2 h at room temperature. Absorbance was measured at a wavelength of 760 nm. A calibration range was established using gallic acid to quantify the total polyphenol concentration. The resultant regression equation from this calibration range was used to calculate the concentration of total polyphenols. This concentration was expressed as micrograms of gallic acid equivalent per milligram of extract (µg EAG/mg extract).

2.6 Total flavonoids

The total flavonoid content of the raw extracts and their corresponding fractions was assessed using the aluminum trichloride method outlined by Shraim et al. [16]. This methodology relies on the reaction between flavonoids and aluminum trichloride in the presence of soda, resulting in the development of a distinctive pink complex with an absorbance peak at 510 nm. In this analytical procedure, a 500 µl aliquot of a properly diluted extract was combined with 2 ml of distilled water and supplemented with 150 µl of a 5% sodium nitrite solution (NaNO2), initiating the process. Following a 6-minute incubation period at room temperature, 150 µl of freshly prepared 10% aluminum chloride solution (AlCl3) was added to the mixture. After an additional 6-minute interval, 2 ml of 4% sodium hydroxide solution (NaOH) was added, and the mixture was allowed to stand for another 6 min. The final volume was adjusted to 5 ml using distilled water. The resultant pink coloration was then quantified by measuring the intensity of the absorption at 510 nm after a 15-minute incubation period. For comparison, a standard range was established using catechin under identical conditions.

2.7 Condensed tannins

Condensed tannins were quantified using the vanilla acid method established by Benzeggouta [17]. In a succinct overview of the procedure, 50 µl of the raw extract was combined with 1500 µl of a solution containing vanilla dissolved in methanol at a concentration of 4% (m/v). The mixture was vortexed to ensure homogenization. Subsequently, 750 µl of hydrochloric acid (HCl) was added and the solution was allowed to react at room temperature for 20 min. To assess the condensed tannin content, absorbance was measured at a wavelength of 550 nm against a blank reference. To establish the calibration curve, a series of concentrations ranging from 0 to 100 µg/ml was prepared from a catechin stock solution. This approach enables the subsequent plotting of the calibration curve, facilitating the determination of condensed tannin concentrations within the samples.

2.8 *In vitro* antioxidant activity

The antioxidant activities of aqueous, methanolic, and acetonic extracts of fresh rosemary and rosemary distillation residue were evaluated using three methods: free radical scavenging (DPPH), ferric reducing antioxidant power (FRAP), and total antioxidant activity (CAT). The antioxidant properties (AOX) of these extracts were compared to those of natural and synthetic AOX, namely BHT and tocopherol.

2.8.1 Free radical scavenging (DPPH) assay

DPPH assay was conducted according to the procedure outlined by Amarowicz et al. [18]. Briefly, 100 µL of aqueous extracts with varying concentrations (5-80 µg/mL) were mixed

with 1300 µL of methanolic DPPH solution (0.1 mM). After 20 min of incubation at room temperature in the absence of light, the reduction in the color intensity was measured at 517 nm. To establish baseline readings, a blank was prepared for each concentration and its absorbance was recorded at 517 nm after 20 min of incubation in the dark at room temperature. α-Tocopherol and synthetic antioxidant BHT were employed as reference materials. Absorbance was determined under the same conditions as the samples and at each concentration.

Radical scavenging activity or the percentage of inhibition (PI), was assessed using the following equation [19]:

$$PI\ (\%) = [(AC - AS) / AC] \times 100 \qquad (2)$$

Where: AC represents the absorbance value in the control, and AS represents the absorbance value in the sample.

2.8.2 Ferric reducing antioxidant power (FRAP)

The FRAP reagent was formulated immediately before use by combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution (dissolved in 40 mM HCl), and 20 mM FeCl3·6H2O at a ratio of 10:1:1 (v/v/v). In the experiment, 0.1 mL of the samples were introduced into a freshly prepared FRAP reagent (3.0 mL). Following the incubation of the reaction mixture at 37 °C for 2 h, the absorbance of the resultant mixture was measured at 593 nm. This measurement was made relative to a blank that encompassed all reagents, except the sample itself [20]. Trolox was used as a benchmark reference, and the results were expressed in terms of micromoles of Trolox per gram of plant dry matter (µM Trolox/g DM).

2.8.3. Conjugated Autoxidizable Triene (CAT)

CAT activity of the extracts was assessed using the phosphomolybdenum method described by Prieto et al. [21]. This approach is based on the reduction of Mo (VI) molybdenum ions in the form of molybdate ions (MoO42 −) to Mo (V) molybdenum (MoO2 +) in the presence of the extract, resulting in a green phosphate/Mo(V) complex under acidic pH conditions.

To perform the procedure, 0.3 mL of each methanolic extract was mixed with 3 mL of the reagent solution composed of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Tubes containing the mixture were incubated at 95°C for 90 min. Following the cooling period, the absorbance of the resultant solutions was quantified at 695 nm, with a reference blank comprising 3 mL of the reagent solution and 0.3 mL of the sample solvent. A blank sample was incubated under the same conditions as the actual sample. The total antioxidant capacity was presented in terms of milligram equivalents of ascorbic acid per gram of dry matter (mg AAE/g DM).

2.9 *In silico* antioxidant activity

In addition to *in vitro* tests, an exploration of the inhibitory potential of the major compounds found in the extract of *Rosmarinus Officinalis* L. (Carnosol (A), Carnosic Acid (B), and Rosmarinic Acid (C)) was undertaken using a molecular docking approach. This involved docking these compounds into the active sites of target proteins, utilizing crystal structures obtained from the Protein Data Bank (PDB) identified by the alphanumeric codes: 2X08 for peroxidase, 4KFQ for receptor GluN1, and 6NGJ for nitric oxide synthase heme.

Standardized procedures were followed for the preparation of both proteins and compounds in readiness for the docking study. Following the docking analysis, comprehensive conformational analyses were performed using Discovery Studio software

(DS). The most noteworthy 2D interactions were extracted and visually depicted in figures, offering a detailed understanding of the binding interactions.

2.10 Statistical analysis

The analysis of variance (ANOVA) was performed to assess the hypothesis suggesting a noteworthy variance in the means across distinct treatments, adhering to a significance level of $p \leq 0.05$. Duncan's mean comparison test was used to unveil the specific group means that exhibited substantial disparities. Furthermore, the standard deviation of the growth rates is meticulously presented, providing valuable insights into the dispersion of the dataset. Statistical analysis was performed using the IBM SPSS Statistics 2021 software.

3 Results and Discussions

3.1 Extraction yield

The hydro-methanolic extract and the aqueous acetone extracts of fresh *Rosmarinus officinalis* (RF) (35.6 ± 2.4% and 29.8 ± 2.1%) had a higher yield than those of the distillation residue (RD) (20.4 ± 1.7% and 15.3 ± 1.3 %), while the filtrate from the distillation residue had a higher yield (59.1 ± 3.6%) than the aqueous extract of fresh Rosemary (45.9 ± 3.1%) (Table 1). It was also noted that the aqueous extracts gave the best extraction yields compared to other hydro-organic solvents. This can be explained by the fact that water is a polar solvent known to extract a wide variety of compounds, including a significant amount of non-phenolic molecules such as proteins and carbohydrates [22].

Table 1. Extraction yield in aqueous and organic extracts of fresh rosemary and distillation residues.

	Extract	**Extraction yield (%)**
Fresh *R. officinalis*	Methanolic extract	35.6 ± 2.4^{b}
	Acetonic extract	29.8 ± 2.1^{bc}
	Aqueous extract	45.9 ± 3.1^{ab}
Distillation residue of *R. officinalis*	Methanolic extract	20.4 ± 1.7^{c}
	Acetonic extract	15.3 ± 1.3^{d}
	Aqueous extract	59.1 ± 3.1^{a}
p value		0.001

Means presented within each column with no common letter(s) are significantly different according to the Duncan test where $p \leq 0.05$.

3.2 Total polyphenol content

The results made it possible to estimate the total polyphenol content in the crude extracts (methanol, acetone, and aqueous extracts) (Figure 1). The methanolic, acetonic, and aqueous fractions contained different amounts of polyphenols. The results also showed that the total polyphenol content varied considerably depending on the solvent used during extraction. The hydro acetone mixture allowed to extract of a higher quantity of polyphenols in the two samples of *Rosmarinus officinalis* (fresh and distillation residue), it contains a higher content in total phenols of around 88.2 ± 1.4 mg EAG / g DM for RF and 65.4 ± 1.9 mg EAG /g DM for RD. The quantity extracted by aqueous methanol is slightly lower than that of acetone in the two samples RF and RD, it reaches respectively 71.1 ± 1.1 mg EAG / g DM and 43.9 ±

1.4 mg EAG / g DM. In contrast, the aqueous fraction of RF and RD showed the lowest polyphenol content 24.0 ± 1.3 mg EAG / g DM in RF and 18.1 ± 1.2 mg EAG / g DM in RD.

The total phenolic data reported in previous studies on *Rosmarinus officinalis* showed values lower than those obtained in this work, with polyphenol levels between 35.3 and 55.5 mg EAG / g DM [23].

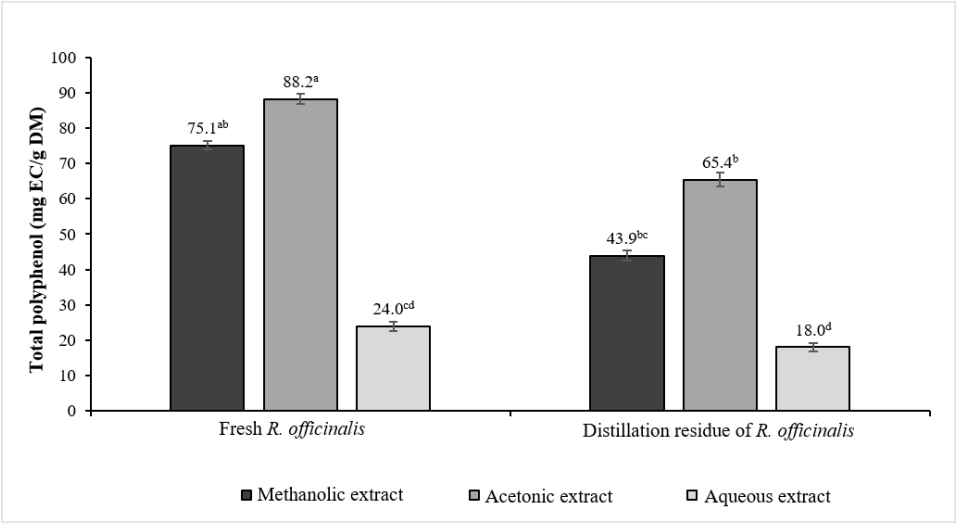

Fig. 1. Total polyphenol content (mg EAG/g DM). Means presented in each bar without a common letter(s) are significantly different according to Duncan's test where p≤0.05.

3.3 Total flavonoid content

The total flavonoid content of the crude extracts was assessed using the aluminum trichloride method, and the calculation was based on the calibration range of catechin. The results were expressed as equivalent milligrams of catechin per gram of dry matter (mg EC/g DM).

Figure 2 illustrates that the total flavonoid content exhibited significant variability depending on the extraction solvent utilized. The quantity of flavonoids found in the hydro-methanolic extract of the two samples RF and RD is the highest, being on average 68.4 ± 1.1 mg EC / g DM and 47.1 ± 1.0 mg EC / g DM respectively. On the other hand, the flavonoid content in the hydro-acetone mixture is slightly lower, 59.3 ± 1.0 mg EC / g DM in RF and 34.8 ± 0.9 mg EC / g DM in RD. The aqueous extracts of RD and RF contain fewer quantity of flavonoids 14.7 ± 0.4 mg EC / g DM for RF and 16.0 ± 0.4 mg EC / g DM for RD. These values are important compared to those reported by Bhagwat et al. [24] (2014), where the quantity of flavonoids contained in the plant significantly exceeded 15 mg EQ/g DM.

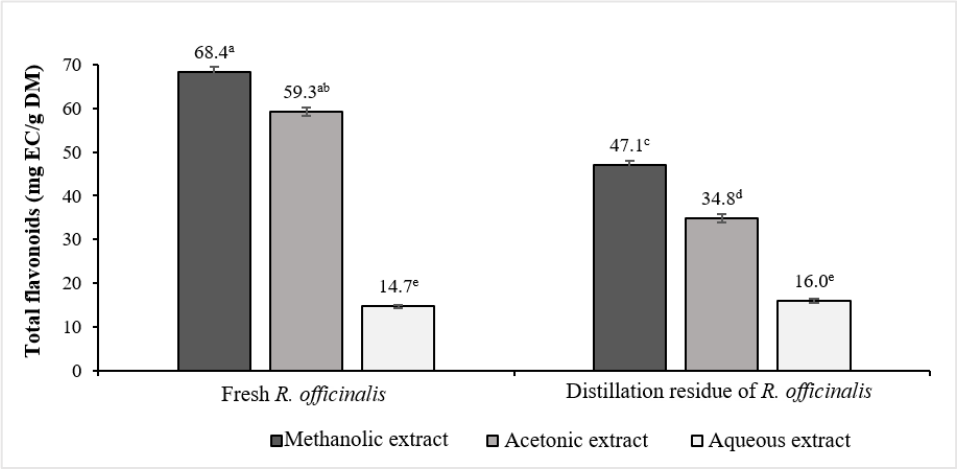

Fig. 2. Total flavonoids content (mg EC/g DM). Means presented in each bar without a common letter(s) are significantly different according to Duncan's test where $p \leq 0.05$.

3.4 Condensed tannins

Figure 3 reveals that the hydro methanolic fraction contains the highest contents of condensed tannins, with a value of 19.3 ± 0.2 mg EC / g DM for RF and 11.8 ± 0.1 mg EC / g DM for RD. On the other hand, the aqueous acetone fractions of RF and RD recorded lower contents; their concentrations reached 17.4 ± 0.2 mg EC / g DM and 10.6 ± 0.1 mg EC / g respectively. While the lowest concentration of condensed tannins was measured in the aqueous extracts 2.7 ± 0.1 mg EC / g DM for RF and 4.8 ± 0.1 mg EC / g DM for RD. Condensed tannins have been shown to exhibit antioxidant properties owing to their antibacterial and antioxidant potential [25]. The notable elevation in condensed tannin levels in the extracts implies a plausible association with the antioxidant attributes of rosemary.

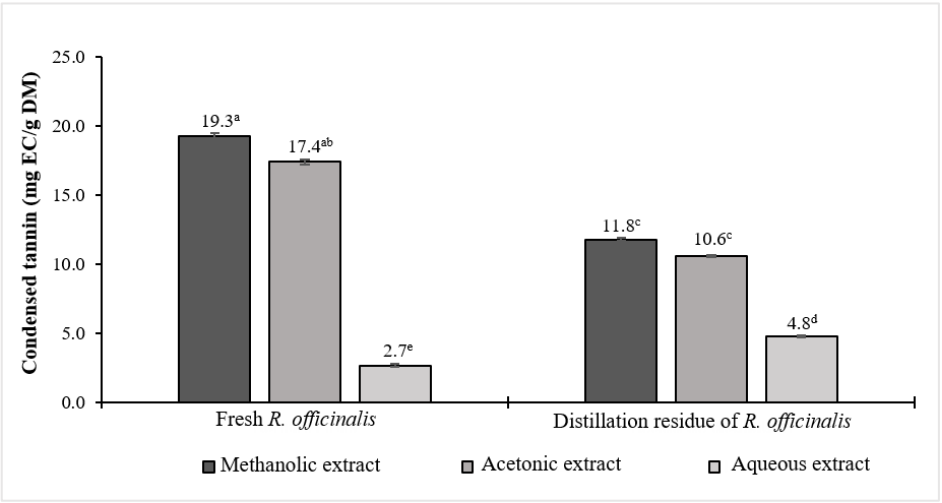

Fig. 3. Condensed tannin content (mg EC/g DM). Means presented in each bar without a common letter(s) are significantly different according to Duncan's test where $p \leq 0.05$.

3.5 Qualitative characterization of the rosemary leaves extracts

The studied extracts revealed the presence of 20 distinct compounds (Tables 2 and 3). These compounds consist predominantly of phenolic diterpenes, flavonoids, and organic acids. Following the results of the determination of total polyphenols, the acetone and aqueous methanol extracts were rich in polyphenols, which is far superior to the organic extracts of the distillation residues. The extraction and accurate quantification of phenolic compounds can pose considerable challenges owing to their intricate chemical composition and divergent solubility across various solvents and extraction techniques, as noted by Das et al. [26]. Shedding light on the subject, Selmi et al. [27] elucidated the existence of 15 distinct compounds within the essential oil of rosemary through meticulous GC–MS analysis. Among these, notable constituents included 1,8-Cineole, transcaryophyllene, and borneol.

Table 2. Phenolic composition of fresh *Rosmarinus officinalis*.

Peak	Polyphenol	Formula	m/z	Acetonic extract	Methanolic extract	Aqueous extract
1	Gallic acid	$C_7H_6O_5$	170.12	+++	+++	+
2	Caffeic acid	$C_9H_8O_4$	180.16	+++	+++	++
3	Syringic acid	$C_9H_{10}O_5$	198.17	+++	+++	ND
4	Vanillic acid	$C_8H_8O_4$	168.14	+++	+++	+
5	Rosmadial	$C_{20}H_{24}O_5$	344.40	+++	+++	Tr
6	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	+	+++	Tr
7	p-coumaric acid	$C_9H_8O_3$	164.05	+	+++	Tr
9	Gallocatechin	$C_{15}H_{14}O_7$	306.27	++	Tr	ND
10	Ferulic acid	$C_{10}H_{10}O_4$	194.18	+++	+	Tr
11	Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.40	Tr	Tr	ND
12	Rosmanol	$C_{20}H_{26}O_5$	346.40	+	+	ND
13	Rutin	$C_{27}H_{30}O_{16}$	610.52	++	++	Tr
14	Rosmarinic acid	$C_{18}H_{16}O_8$	360.31	+++	+++	+
15	Quercetin	$C_{15}H_{10}O_7$	302.24	+	Tr	ND
16	Apigenin	$C_{15}H_{10}O_5$	270.05	+	Tr	ND
17	Thymol	$C_{10}H_{14}O$	150.22	Tr	Tr	ND
18	Epirosmanol	C20H26O5	346.42	+	+	ND
19	Carnosol	$C_{20}H_{26}O_4$	330.40	+++	+++	ND
20	Carnosic acid	$C_{20}H_{28}O_4$	332.42	+++	+++	Tr

Quantity: (+++) Very significant; (++) Significant; (+) Average; (Tr) Trace; (ND) Not detected.

Table 3. Phenolic composition of distillation residue of *Rosmarinus officinalis*.

Peak	Polyphenol	Formula	m/z	Acetonic extract	Methanolic extract	Aqueous extract
1	Gallic acid	$C_7H_6O_5$	170,12	++	++	++
2	Caffeic acid	$C_9H_8O_4$	180,16	++	++	++
3	Syringic acid	$C_9H_{10}O_5$	198,17	++	++	Tr
4	Vanillic acid	$C_8H_8O_4$	168,14	++	++	+
5	Rosmadial	$C_{20}H_{24}O_5$	344,40	++	++	Tr
6	Chlorogenic acid	$C_{16}H_{18}O_9$	354,31	+	++	Tr
7	p-coumaric acid	$C_9H_8O_3$	164,05	+	++	Tr
9	Gallocatechin	$C_{15}H_{14}O_7$	306,27	Tr	Tr	ND
10	Ferulic acid	$C_{10}H_{10}O_4$	194,18	+	Tr	Tr
11	Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.40	Tr	Tr	ND
12	Rosmanol	$C_{20}H_{26}O_5$	346.40	+	+	ND
13	Rutin	$C_{27}H_{30}O_{16}$	610,52	+	+	ND
14	Rosmarinic acid	$C_{18}H_{16}O_8$	360,31	++	++	++
15	Quercetin	$C_{15}H_{10}O_7$	302,24	+	Tr	Tr
16	Apigenin	$C_{15}H_{10}O_5$	270,05	+	Tr	Tr
17	Thymol	$C_{10}H_{14}O$	150,22	ND	ND	ND
18	Epirosmanol	$C_20H_{26}O_5$	346.42	+	+	Tr
19	Carnosol	$C_{20}H_{26}O_4$	330.40	++	++	Tr
20	Carnosic acid	$C_{20}H_{28}O_4$	332,42	++	++	Tr

Quantity: (+++) Very significant; (++) Significant; (+) Average; (Tr) Trace; (ND) Not detected.

3.5.1. Phenolic diterpenes

Five phenolic diterpenes were identified in the aqueous and hydromethanolic acetone extracts of fresh rosemary and distillation residue, namely rosmadial, rosmanol, epirosmanol, carnosol, and carnosic acid (Figure 4).

Wenkert et al. [28] described a carnosic acid concentration of about 0.35% in dried leaf samples. Zhang et al. [29] investigated the phenolic composition and antioxidant activity of rosemary extracts from different geographical origins. They found that carnosic acid content ranged from 0.7% to 2.1% in the analyzed samples. Furthermore, the phenolic composition of rosemary depends on the species, variety, plant growth conditions, sample treatment, and extraction method.

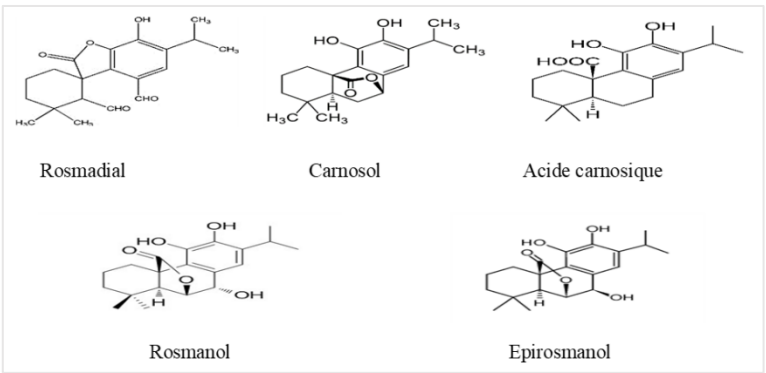

Fig. 4. Structures of phenolic diterpenes identified in rosemary extracts.

3.5.2. Phenolic acids

Nine phenolic acids were identified in rosemary: caffeic acid, vanillic acid, gallic acid, chlorogenic acid, syringic acid, ferulic acid, dicaffeoylquinic acid, p-coumaric acid, and rosmarinic acid (Figure 5). These results are in agreement with those of previous studies [30-31].

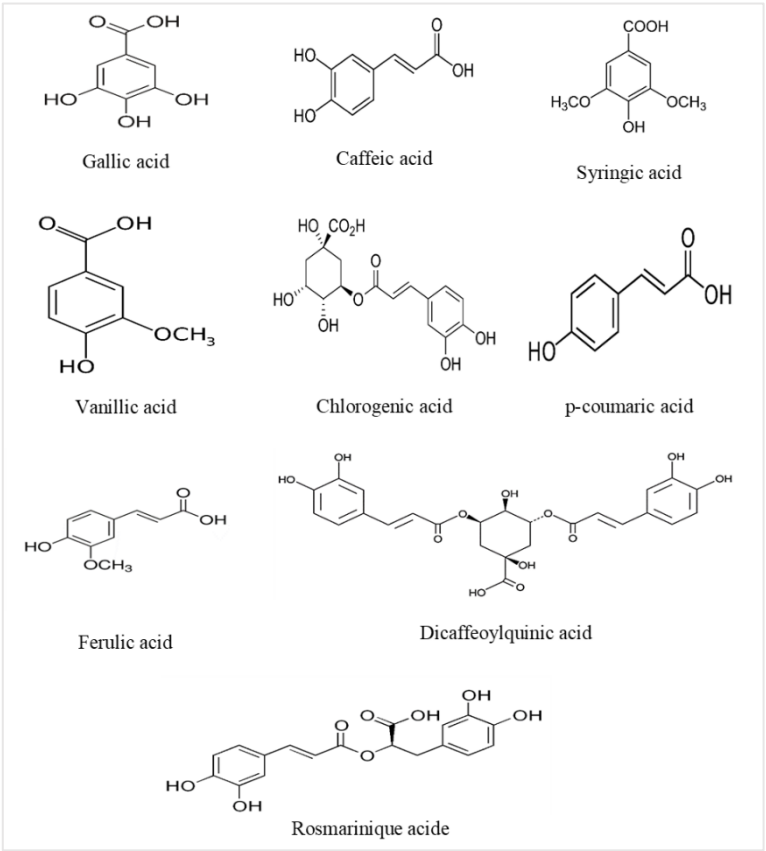

Fig. 5. Structures of phenolic acids identified in rosemary extracts.

3.5.3. Flavonoids

The presence of four flavonoids in the extracts of the fresh rosemary and distillation residue was also observed. These flavonoids were distributed into flavones, such as apigenin, and flavonols, including quercetin, gallocatechin, and rutin (Figure 6).

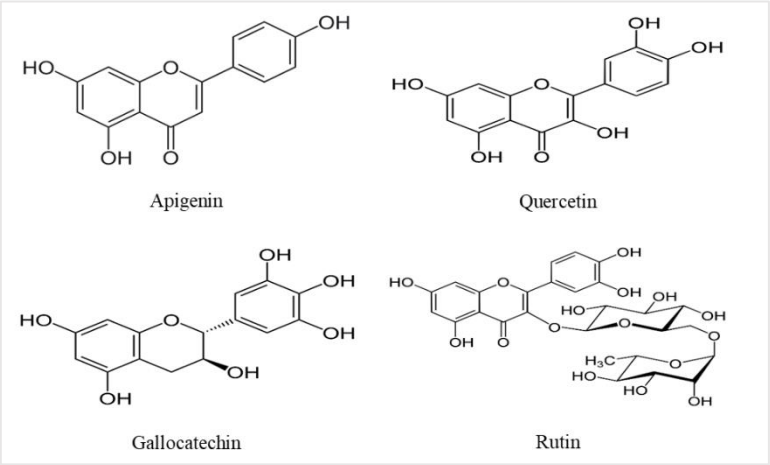

Fig. 6. Flavonoids identified in rosemary extracts.

In a study conducted by Mena et al. [32], meticulous analysis highlighted the presence of 24 distinct flavonoids. Although these compounds are predominantly composed of flavones, the array of compounds includes flavonols and flavanones. Moreover, through LC-ESI-MS/MS analysis, Hossain et al. [33] revealed 38 phenolic compounds present in solid/liquid extracts derived from five Lamiaceae species: rosemary, sage, oregano, basil, and thyme. It is worth emphasizing that among this array of compounds, flavonoids emerged as the most prolific, encompassing a remarkable number of 17 distinct constituents.

Given the insights derived from the aforementioned outcomes, it is evident that the extraction of phenolic compounds constitutes a pivotal phase in the development of the active ingredients. The efficacy of extraction is inherently tied to the choice of the extraction solvent. Thus, the strategic selection of an appropriate solvent system remains a fundamental step in the optimization of polyphenol, flavonoid, and other antioxidant compound extraction, as underscored by Chaves et al. [34].

3.6 *In vitro* antioxidant activity

3.6.1. Free radical scavenging (DPPH) assay

Table 4 presents the results of the evaluation of antioxidant activity using the DPPH method based on the IC50 value. This value signifies the quantity required by each extract to neutralize 50% of the free radicals and inversely varies with the percentage of free radical inhibition. A notable variation was observed, with the aqueous extract of fresh rosemary exhibiting the highest IC50 (indicating lower antioxidant activity). However, the methanolic and acetone extracts revealed values similar IC50 to that of the reference product (BHT). A proportional increase in inhibition percentage with increasing concentrations was observed (Figure 7).

Table 4. Antioxidant activity of rosemary extracts using a free radical scavenging assay (DPPH).

	Extract	**IC50 (µg/ml)**	**Inhibition of DPPH (%)**
Fresh *R. officinalis*	Methanolic extract	21±0.3^{c}	86±1.4
	Acetonic extract	19±0.7^{c}	85±1.8
	Aqueous extract	60±0.9^{a}	63±1.3
Distillation residue of *R. officinalis*	Methanolic extract	40±0.5bc	79±1.6
	Acetonic extract	32±0.7^{cd}	79±1.5
	Aqueous extract	45±0.9^{b}	63±1.4
α-Tocopherol		13±0.3^{d}	92±1.6
BHT		20±0.1^{c}	83±0.1
p-value		0.003	ns

Means presented within each column with no common letter(s) are significantly different according to the Duncan test where $p \leq 0.05$. ns: not significant.

Rosemary extract exhibits significant antioxidant activity, primarily attributed to two crucial antioxidant components belonging to the classes of phenolic acids, flavonoids, and diterpenoids, namely carnosol (C20H28O4) and carnosic acid (C20H28O4). Carnosol and carnosic acid are phenolic diterpenes responsible for the primary antioxidant activity of rosemary extract [8,35]. Furthermore, other studies have indicated that the antioxidant properties of rosemary stem are primarily due to its high isoprenoid quinone content, which acts as a scavenger of reactive oxygen species (ROS) [36].

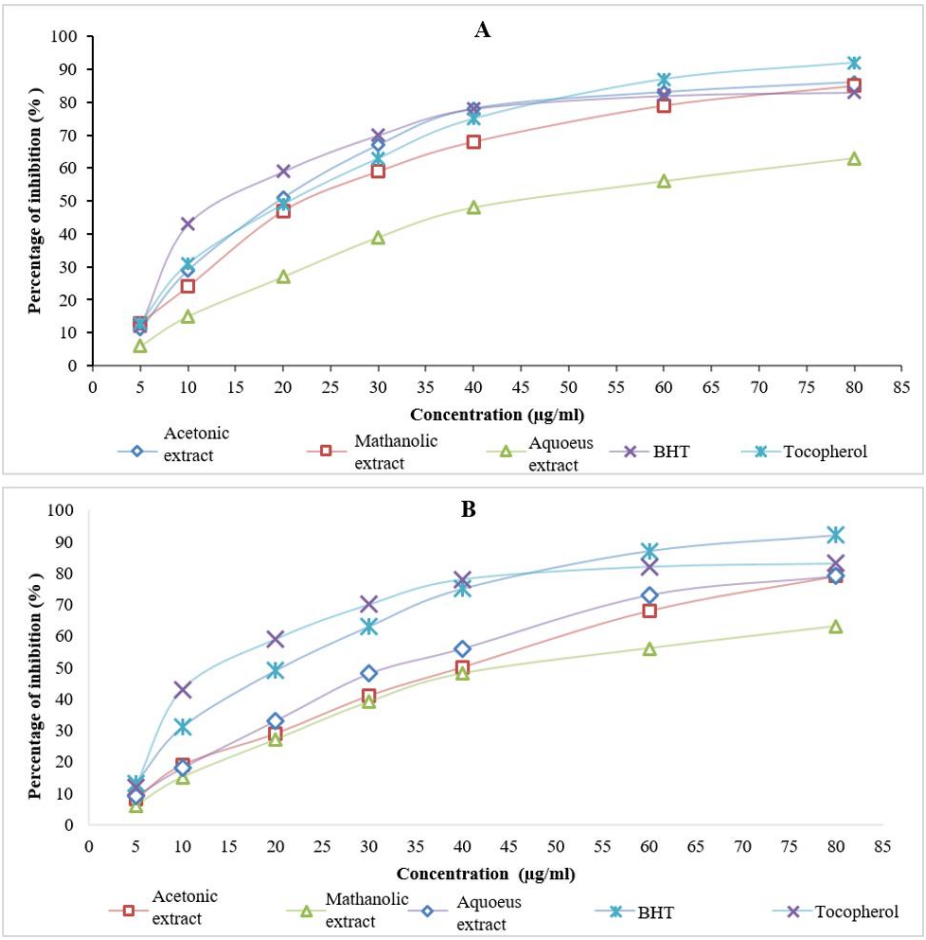

Fig. 7. Free radical-scavenging capacities (%) of fresh rosemary (A), distillation residue (B) extracts and standard antioxidants (BHT and Tocopherol).

3.6.2. Ferric reducing antioxidant power (FRAP)

The efficacy of natural plant extracts in reducing Fe3+ to Fe2+, as measured by antioxidant power, is presented in Figure 8, expressed in µM Trolox equivalents (TE) per gram of dry matter (DM). The data demonstrated that all extracts exhibited strong and comparable ferrous-reducing antioxidant capacities, ranging from 80.2 µM TE/g to 175.0 µM TE/g.

The antioxidant activity of the aqueous extracts was the highest in the distillation residues, whereas it was the lowest in the case of fresh rosemary (Figure 8). All extracts exhibited high reducing power, indicating the presence of electron-donating compounds capable of reacting with Fe3+ ions and converting them into more stable products. The reducing power of acetone and aqueous methanol extracts surpasses that reported by Lorena Martínez et al. [37], which ranges from 61.3 to 73.8 µM TE/g DM.

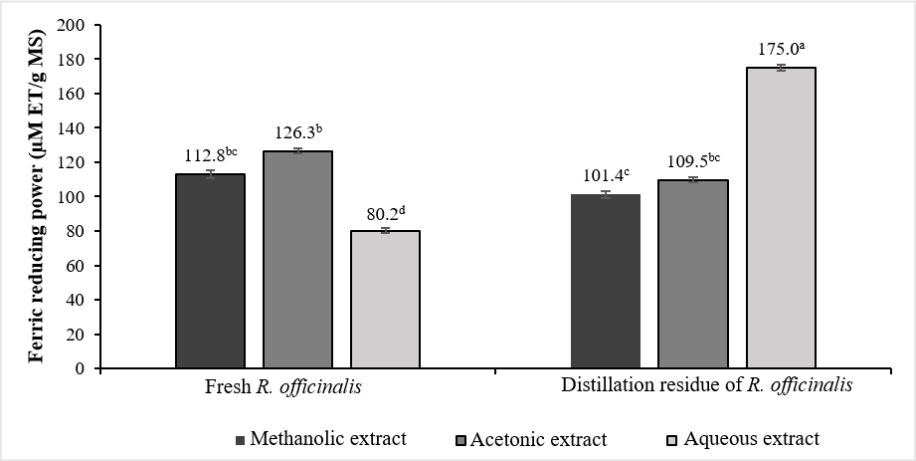

Fig. 8. Antioxidant activity of rosemary extracts using ferric reducing antioxidant power (FRAP). Means presented in each bar without a common letter(s) are significantly different according to Duncan's test where $p \leq 0.05$.

3.6.3. Conjugated Autoxidizable Triene (CAT)

Figure 9 demonstrates that the investigated extracts displayed diverse total antioxidant activities, which were influenced by the type of solvent employed for extraction.

Acetone extracts exhibit the highest total antioxidant capacities, with values of 156.8 and 106.4 mg AAE/g DM for fresh rosemary and distillation residues, respectively. In contrast, aqueous extracts displayed lower activity, with values of 30.6 and 40.7 AAE/g DM, respectively. Unlike other tests, CAT not only quantifies the contribution of polyphenolic antioxidant activity but also other antioxidant compounds, such as vitamins [38].

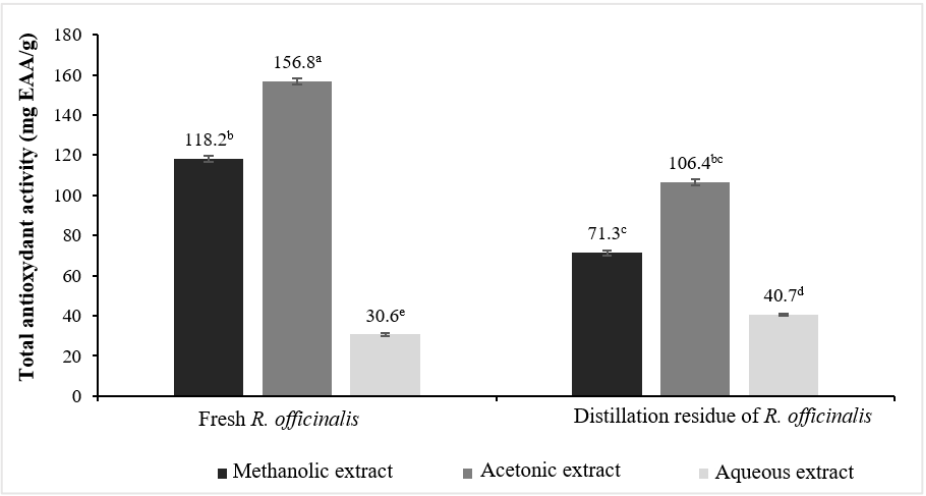

Fig. 9. Antioxidant activity of rosemary extracts using Conjugated Autoxidizable Triene (CAT). Means presented in each bar without a common letter(s) are significantly different according to Duncan's test where $p \leq 0.05$.

3.7 *In silico* antioxidant activity

The investigation involved the molecular docking of three compounds, carnosol (A), Carnosic Acid (B), and Rosmarinic Acid (C), into the active sites of the target proteins to assess their inhibitory potential as antioxidants. The selected molecular targets were peroxidase (PDB ID: 2X08), GluN1 (PDB ID: 4KFQ), and nitric oxide synthase heme (PDB ID: 6NGJ).

For peroxidase (PDB ID = 2X08), carnosol (A) interacted with key residues such as Glu35, Asp37, and Arg184, forming conventional hydrogen bonds, and Tyr42 and Ala36 through hydrophobic interactions (Figure 10, [I] A). The second compound engaged in hydrogen bonding with Gly41, with π-π stacked interactions with Tyr42 and π-alkyl interactions with VAL45, His181, Ile40, and ALA36 (Figure 10, [I] B). Rosmarinic Acid (C) displayed conventional hydrogen-bonding interactions with Tyr42, Lys90, Glu35, and Lys179, along with hydrophobic interactions with His181, Val45, Pro44, and Ala36 (Figure 10, [I] C).

Upon docking into the GluN1 receptor (PDB ID = 4KFQ), carnosol (A) demonstrated hydrogen bonding interactions with Gly93 and Arg131, and pi-alkyl interactions with Lys91, Trp223, Phe92, and Val227 (Figure 10, [II] A). Carnosic Acid (B) interacted with Gly93, Ser180, and Arg131 through conventional hydrogen bonding, accompanied by π-π interactions with Lys91, Ile183, and Val181, as well as π-lone interactions with Phe92 (Figure 12, [II] B). Rosmarinic Acid (C) engaged in hydrogen bonding with Asp224, Asn107, Thr94, Gly93, and Ser180, as well as hydrophobic interactions with Phe92, Trp223, and Lys91 (Figure 10, [II] C).

In the case of nitric oxide synthase heme (PDB ID = 6NGJ), carnosol (A) interacted through hydrogen bonding with Val416, G417, Cys415, and Glu592, and displayed π-alkyl interactions with Trp409, Trp587, Val567, and Phe584 (Figure 10, [III] A). Carnosic Acid (B) exhibited hydrogen interactions with Val416 and Cys415, hydrophobic interactions with Phe704, Ala412, Trp678, and Phe584, and π-sigma interactions with Trp409 (Figure 10, [III] B). Rosmarinic Acid (C) engaged in hydrogen bonding with Gly586, Pro565, and Glu592, and hydrophobic interactions with Phe584, Cys415, Ala654, and Val649 (Figure 10, [III] C).

Carnosol, a naturally occurring diterpene found in *Rosmarinus officinalis* L., has noteworthy antioxidant properties. Carnosol, along with other diterpenes, accounts for more than 90% of the antioxidant components present in rosemary extracts [35]. The effectiveness of carnosol as an antioxidant is linked to its active chemical composition, which is characterized by ortho-phenolic groups [39]. Moreover, Bouammali et al. [40] showed that both rosmarinic acid and carnosic acid effectively prevent oxidative stress and protect against oxidative damage through diverse mechanisms. These include scavenging free radicals, inhibiting lipid peroxidation, and modulating antioxidant enzyme activities. The intrinsic antioxidant properties of these compounds make them valuable agents for preventing diseases associated with oxidative stress such as cancer. Collectively, carnosol, rosmarinic acid, and carnosic acid have emerged as pivotal allies in the ongoing battle against various health challenges.

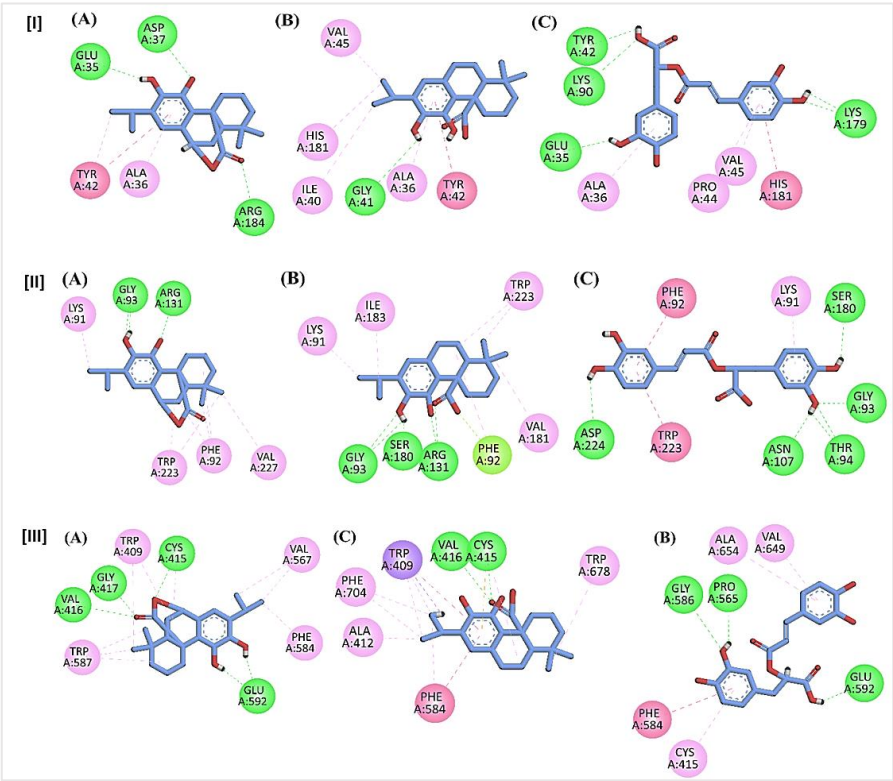

Fig. 12. 2D Interaction Plots of Carnosol (A), Carnosic Acid (B), and Rosmarinic Acid (C) with peroxidase (pdb id:2x08), receptor GluN1 (pdb id:4KFQ) [II], and with nitric oxide synthase heme (pdb id:6NGJ) [II].

4 Conclusion

The results of this study underscore the importance of extracting phenolic compounds from rosemary, revealing a rich composition of polyphenols, flavonoids, and tannins that significantly contribute to the diverse biological activities of the plant, particularly its antioxidant properties. Chemical profiling revealed potent antioxidant molecules, such as rosmarinic acid, carnosol, and carnosic acid, emphasizing their pivotal roles in therapeutic and preservative effects. Despite substantial quantities of phenolic compounds with notable antioxidant activities in rosemary extracts, each extract exhibited distinct behavior, likely influenced by variations in the chemical composition.

Moreover, a molecular docking investigation explored the antioxidant potential of three specific compounds, carnosol, Carnosic Acid, and Rosmarinic Acid, against selected target proteins (peroxidase, GluN1, and nitric oxide synthase heme). These compounds demonstrate unique interactions with key residues in the active sites of peroxidase and GluN1, involving hydrogen bonding and hydrophobic interactions. Similar specific bonding patterns observed with nitric oxide synthase heme suggested potential antioxidant activities, highlighting the diverse interactions and utility of the compounds as antioxidants against the studied protein targets.

These findings provide a robust foundation for further exploration of rosemary applications in various industries, ranging from food and pharmaceuticals to healthcare, owing to its rich phenolic content. The identified bioactive compounds present promising

opportunities for the development of novel antioxidants and therapeutic agents, highlighting the multifaceted potential of rosemary across diverse fields.

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Author Contributions

Conceptualization and methodology, K.E. and T.E.; validation, M.B.I. and A.Br.; formal analysis; A.Bt. and K.E., investigation, K.E. and T.K.; writing-original draft preparation, K.E., A.Bt., and T.K.; writing-review and editing, A.Bt., N.E., M.E.; visualization, A.Bt., and M.B.I.; supervision, A.Br.; All authors have reviewed and consented to the final version of the manuscript for publication.

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