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STUDY OF ANTIOXIDANT, BIOLOGICAL ACTIVITY AND DOCKING STUDY FOR PHENOLIC ACIDS IN *ROSMARINUS OFFICINALIS* L. CRUDE EXTRACT

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ABSTRACT

Plants are a mine of many medical substances, making them a pharmacy used by humans to treat any disease or develop new drugs. This research rosemary was chosen to study the antioxidant activity, Biological activity against bacteria, fungi and combine this study with docking software (MOE) to demonstrate the biological activity. The results show the valuable antioxidant activity of rosemary extract using radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging action with an IC50 value of 35.45% ± 1.22 µg/ml. Biological activity of rosemary extract detect by using bacterial and fungal isolates, and MIC values for Bacterial isolate were (0.5, 0.4, 0.4, 0.5) mg/ml for (*Escherichia coli*, *Shigella sp*., *Staphylococcus aureus*, and *Bacillus subtilis*) respectively, While 0.5 mg/ml for *Candida albicans* and 0.4 mg/ml for *Aspergillus sp*. For docking study, three phenolic acids were used to explain the biological activity against bacteria and fungi by using two proteins for bacteria which give good binding energy when interaction with penicillin-binding protein about (-5.99311018, -5.67873335, and -5.67580652) for (rosmarinic acid, carnosic acid, and carnosol respectively) while binding energy for interaction with outer membrane protein A give (-4.84447241, -4.58120823, and -4.50296259) (rosmarinic acid, carnosic acid, and carnosol respectively). For fungi also two proteins were chosen EXO-beta-(1,3)-glucanase protein and Crh transglycosylases protein and the energy binding score were (-7.03097296, -6.47496843, and -5.67503548), (-4.57932281, -5.71598148, and -4.50296259) for (rosmarinic acid, carnosic acid, and carnosol respectively).

Keywords: *Rosmarinus officinalis* L., Phenolic acids, Rosmarinic acid, Carnosic acid, Carnosol, Antioxidant

INTRODUCTION

Mint Family *(Lamiaceae)* one of the most influential families in the plant kingdom because it includes many genera that have medical uses, such as Rosemary, Thyme, and Peppermint; throughout the ages, humans used many plants in this family as a source of food, flavors, fragrances, and medicines, even today a large number of people exploits this family in traditional medicine with the reason of containing many active constituents acting individually, additively or in synergy to improve health (Carović et al 2016). *Rosmarinus officinalis* L., commonly known as rosemary, belongs to the Lamiaceae family, is one of the essential species recently. The genus *Rosmarinus* has been merged into the genus *Salvia* in phylogenetic analysis. It is an aromatic plant used to prepare traditional food and folk medicine for its therapeutic properties as oral preparation to relieve renal colic. rosemary has antifungal, antiviral, antibacterial, anti-inflammatory, antitumor, and antioxidant activities and in pharmaceutical industries used in the preparation of massage oils and ointments for massage and aromatherapy purposes (German et al., 2016; Macedo et al., 2020). The therapeutic effect of rosemary came from its active constituents, which include essential oil, flavonoids such as hesperidin, hispidulin, and diosmin, and several types of phenolic acids, for example (carnosol, rosmarinic acid, carnosic acid); all these constituents have a specific activity to give the medical effect of this plant (Nieto et al., 2018). Phenolic acids one of the most essential compounds in rosemary, which are described as phenols that possess one carboxylic acid functionality. They are responsible for free radical scavenging, metallic ions chelation, and changing enzymatic activity. Moreover, these compounds exhibit antiviral activity, for instance, rosmarinic acid, anti-inflammatory action, for example, a mixture of esters of benzoic and cinnamic acids (Robbins 2003; Arceusz et al., 2013). Phenolic acids can be extracted from fresh, frozen, or dried plant biomass. Usually, however, plant biomass is air-dried or freeze-dried and then milled into a homogeneous powder before phenolic acid extraction. The extraction from dry biomass is favored over-extraction from fresh biomass because enzymes in fresh cells can potentially degrade phenolic acids, the best solvent to prepare crude extract contain phenolic acids consist of a combination between ethanol, methanol, and acetone with water, the percent of the resulted solution is 20%–50%(Al Jitan et al., 2018). The present study aimed to evaluate antioxidant activity, antibacterial and antifungal activity of phenolic acids in the crude extract of *Rosmarinus officinalis* L. and used molecular docking study to explain the antibacterial and antifungal activity of some phenolic acids in rosemary.

MATERIALS AND METHODS

Plant collection: The plant was collected from a plantation in Basra province and classified as *Rosmarinus officinalis* L. Plant leaves were collected and drying in the shade. Then we grind them to a fine powder by the electric mill.

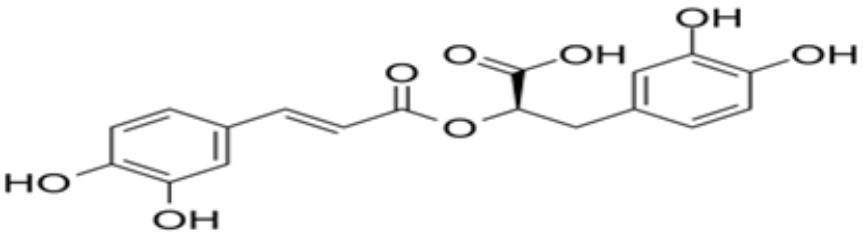
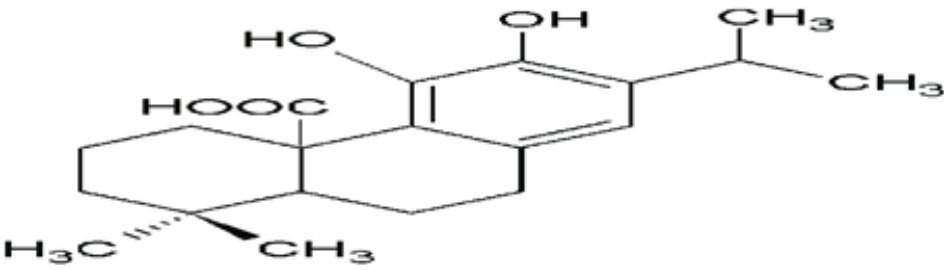
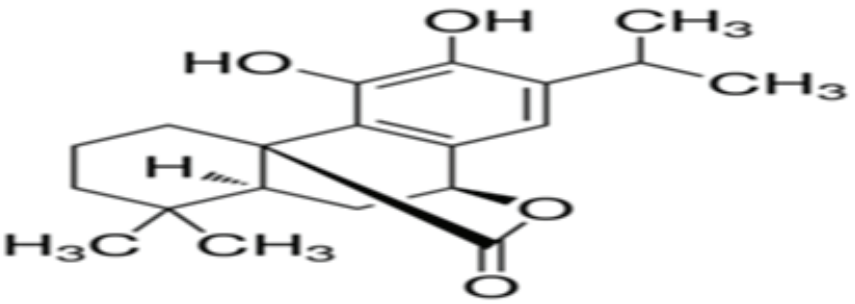
Table 1: Crystallographic properties of selected bacterial and fungal protein

Protein	PDB Code	Classification	Organism	Expression system	Resolution	Method	Total structure weight (kDa)	chain
Penicillin-Binding Protein 3 (PBP3)	3VSL	Penicillin-Binding Protein	*Staphylococcus aureus* subsp.aureus MW2	*Escherichia coli*	2.40 Å	X-Ray Diffraction	145.06	A, B
Outer membrane protein A (OMPA)	1BXW	Membrane protein	*Escherichia coli* BL21(DE3)	*Escherichia coli* BL21(DE3)	2.50 Å	X-Ray Diffraction	19.20	A
EXO-beta-(1,3)-glucanase	1EQC	Hydrolase	*Candida albicans*	*Saccharomyces cerevisiae*	1.85 Å	X-Ray Diffraction	45.46	A
Crh transglycosylases	6IBW	Hydrolase	*Aspergillus fumigates* Af293	*Escherichia coli* BL21-Gold(DE3) pLysS AG	2.80 Å	X-Ray Diffraction	55.70	A, B

Table 2: Lipinski's physicochemical parameter for selected phenolic acids from rosemary ethanolic extract

Compounds	Mol. Weight g/mol	h_logP	h_logS	a_acc	a_don	TPSA (A°)	b_rotN	lip_druglike
Rosmarinic acid	361.3259	-9.9706	*3.53066*	*5*	3	60.6899	1	1
Carnosic acid	332.4400	4.81273	*-4.4723*	*4*	4	77.7600	2	1
Carnosol	330.4239	4.40772	*-4.5503*	*3*	2	66.7600	1	1

Table 2: Chemical structure of selected phenolic acids in rosemary ethanolic

Phenolic acids	Chemical Structure
Rosmarinic acid	
Carnosic acid	
Carnosol	

Bacterial isolates	Inhibition zone (mm)
Escherichia coli	10
Staphylococcus aureus	24
Bacillus subtilis	26
Shigella sp.	11
Fungal isolates	**Inhibition zone (mm)**
Candida albicans	26
Aspergillus sp.	25

Table 3: Inhibition zone for rosemary extract against bacterial and fungal isolates

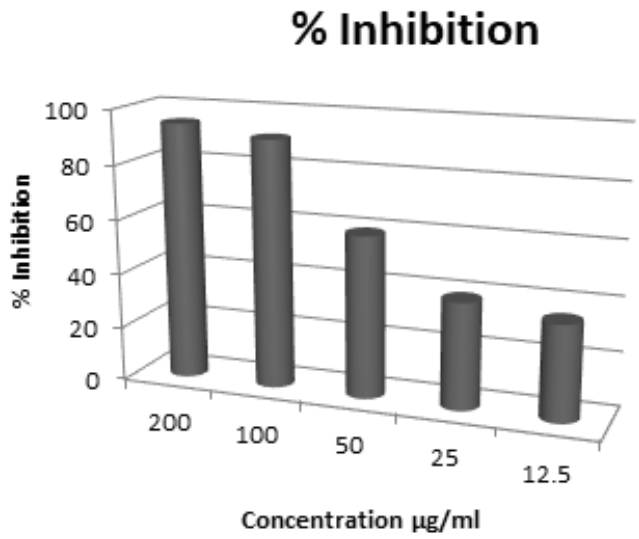


Figure 1: the DPPH inhibition% for each concentration of Rosemary extract

Extraction method: To prepare crude extract to contain phenolic acid, the extraction process was accomplished by stirring 10g of leaves powder with 100ml of 50% ethanol for 3h, and the extraction temperature was 65 ℃. The extract was then filtered by using Whatman number 4 filter paper. Then freed of solvent by evaporation at room temperature, the dried crude extract was stored at -20C (KIDO-2018).

Determination of the antioxidant activity of rosemary extract by radical scavenging activity of the DPPH:

The antioxidant activity of rosemary extract was measured as a radical scavenging capacity of radical DPPH. The Erenler et al. 2016 method with a little modification was followed to accomplish the experiment (9). 0.5ml of 0.2 mM DPPH methanolic solution was added to 2.5 ml of the extracts with concentration (12.5-200) µg/ml. Then the mixture was shaken vigorously, and the tubes were enclosed tightly and allowed to stand for 30 min in the dark. Absorbance against blank samples was measured at 517 nm and compared to the calibration curve of ascorbic acid. The assay was carried out in triplicate. The below formula calculated the DPPH radical inhibition percentage:

$$\% \text{ inhibition} = [(\text{Control- Test})/\text{control}] \times 100$$

the plotted graph of scavenging activity against the different concentrations of rosemary extracts can be used to determine the IC_{50} value, which is defined as the total antioxidant needed to decrease the initial DPPH concentration 50%. The reference compound as Ascorbic acid.

Bacterial and fungal isolates	MIC mg/ml
E. coli	0.5
shigella	0.4
S. aureus	0.4
Bacillus subtilis	0.5
Candida albicans	0.5
Aspergillus	0.4

Table 4: MIC value for bacterial and fungal isolates

Qualitative of antibacterial and antifungal activity of rosemary extract

The antimicrobial activity of rosemary crude extract was estimated qualitatively by using the agar well diffusion technique. The concentration of 24 mg/ml was used as a concentration detector for antibacterial activity assay Fatima et al., 2009. Local bacterial and fungal isolates were tested. The bacterial isolates include two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two-gram negative bacteria (*Escherichia coli* and *Shigella sp.*), which were provided by the microbiology laboratory, Pharmacy College, Basrah University). In addition to two different species of fungi, *Candida albicans* and *Aspergillus sp.* (supplied by the central laboratory, Veterinary Medicine College, Basrah University). To reach the stationary growth phase, bacteria and fungi were incubated at 37°C and 25°C, respectively, for 24 h in Nutrient Broth (NB, Difco, MD, USA). A 10^8 Colony Forming Unit (CFU) per ml was achieved by used McFarland 0.5. Cefotaxime (2 mg/ml) was used as a positive control, and pure DMSO was used as a negative control. Each plate (Mular Hinton agar) was inoculated with only one microorganism. Three wells per plate were made using an 8 mm sterile cork borer. The three wells were inoculated with 100 µl of rosemary extract solution, positive and negative control, respectively. Then, bacterial and fungal plates were incubated for 18 h at 37°C and 25°C, respectively. The diameter of clear zones around each well was measured, showing no bacterial or fungal growth (Fatima et al., 2010).

Minimum Inhibitory (MIC) Assay

The minimum inhibitory concentration (MIC) was obtained by using well diffusion agar as described by Pereira et al. 2008 with a little modification. Briefly, Petri dishes containing MH agar were seeded by six

Phenolic acids	The score of binding (Kcal/mol)
Binding score with PBPs protein	
Rosmarinic acids	-5.99311018
Carnosic acid	-5.67873335
Carnosol	-5.67580652
Binding score with OMPA protein	
Rosmarinic acids	-4.84447241
Carnosic acid	-4.58120823
Carnosol	-4.50296259

Figure 3: 2D diagram illustrate theoretical interaction from docking software between selected phenolic acids in rosemary extract and PBPs protein

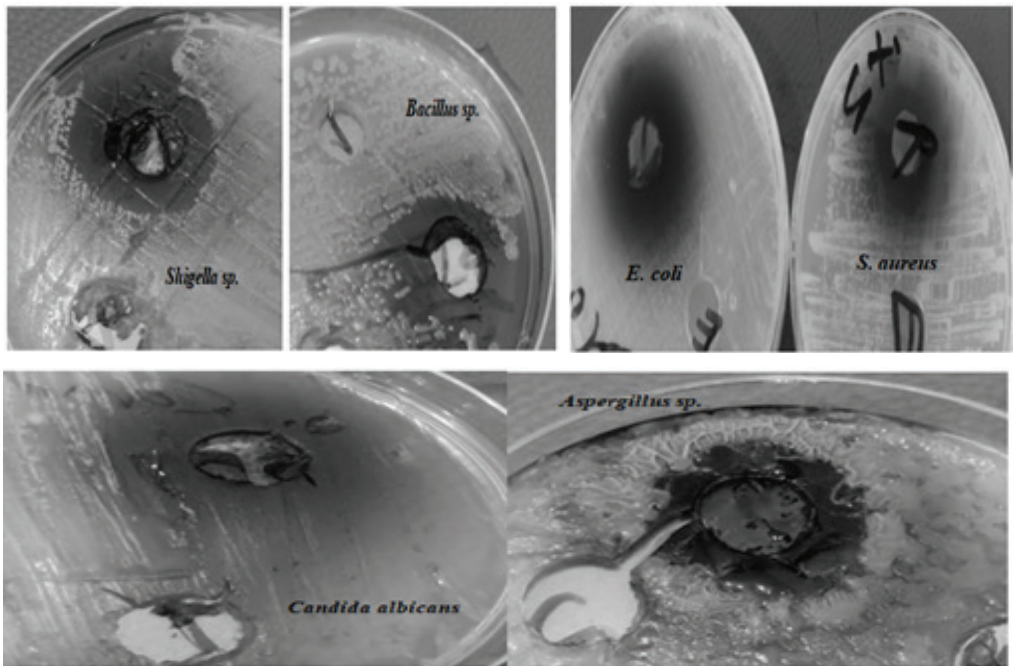


Figure 2: Antibacterial and antifungal activity of rosemary

microorganisms (two gram-positive, two-gram negative bacteria, and two fungi). Eight concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mg/mL) of rosemary extract was tested against these microorganisms. The inoculated plates were incubated at 37°C for 18-24 hours. Then, the antimicrobial activity was calculated by measuring the no-growth zone around each well. The MIC values were documented as the lowest concentrations of the extract showing a clear zone. Solvent (DMSO) was used as a negative control. Positive controls were used for bacteria (Amin et al., 2013).

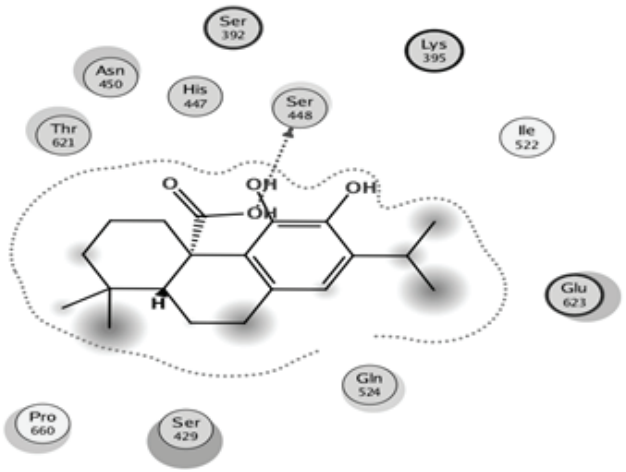
Molecular docking study for some phenolic acids in rosemary extract as antibacterial and antifungal agents

In this study, three phenolic acids in rosemary extract were chosen (rosmarinic acid, carnosic acid, and carnosol) (Kuhlmann 2006). Two proteins in gram-positive and gram-negative bacteria [3VSL Penicillin-Binding Protein 3 (PBP3) and 1BXW Outer membrane protein A OMPA] (13,14), while for fungi, two proteins in *Candida albicans* (1EQC EXO-beta-(1,3)-glucanase) and *Aspergillus fumigates* (6IBW Crh transglycosylases) (Fang et al., 2019; Cutfield et al., 2000) were downloaded from the protein data bank. Crystallographic properties of these proteins illustrated in table 1, Molecular Operating Environment (MOE software) was used for docking study, the energy for each protein was minimized and (Amber 10) was used as force field energy then the suitable active site was detected for docking with selected phenolic acids which download as SDF formate from PubChem. Lipinski's physicochemical parameters rule for each selected phenolic acids showed in table 2 and also chemical structure illustrated in table 3 (Bouchentouf et al., 2019).

RESULTS AND DISCUSSION

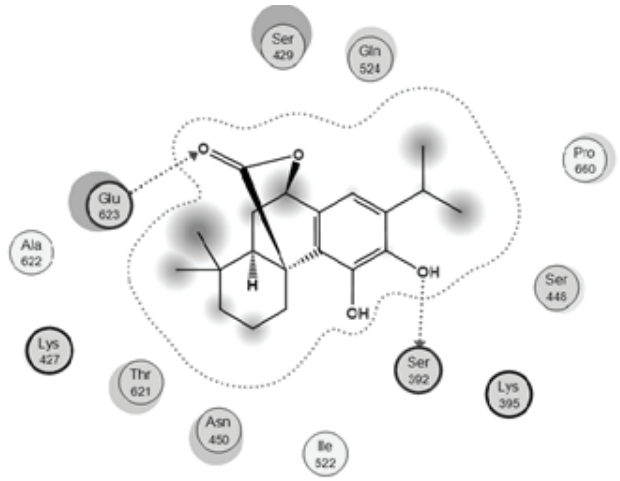
Antioxidant activity of rosemary extract by radical scavenging activity of the DPPH: In this study, the antioxidant capacity of rosemary extract was designated in Figure 1. Which showed the DPPH radical scavenging activity over the range of (12.5 –200) µg/ml concentration, and the IC_{50} of DPPH scavenging activities extracts was 35.45% ± 1.22 µg/ml.

Rosemary extract showed a valuable antioxidant activity, and that is according to the presence of different phenolic acids. However, rosemary riches by many active constituents, but phenolic acids have a particular effect as antioxidant activity, and this approved by previous researches, which illustrated that the phenolic compounds existing in the extracts of rosemary act as primary antioxidants when reacting with the lipid and hydroxyl radicals to turn them into stable products, carnosic acid and carnosol act as potent scavengers of peroxyl radicals. The antioxidant activity of phenolic acids came from the presence of hydrogen-donating groups that can react with oxidants to form resonance-stabilized phenoxyl radicals and provide antioxidant properties (Liuet al., 2020).



The interaction between carnosic acid and PBPs proteins:

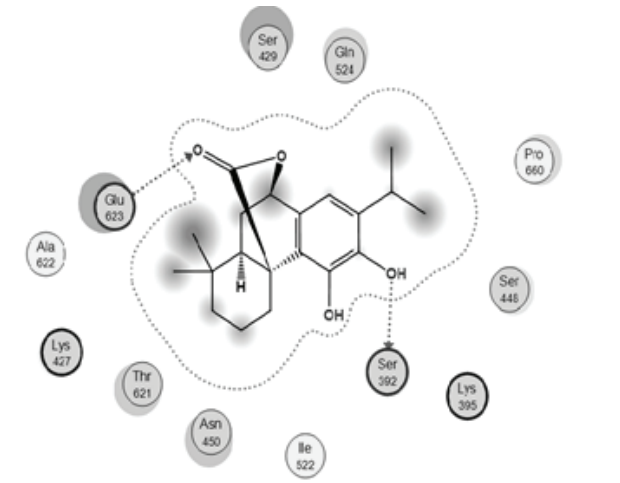
The amino acid SER 488 (H-donor) with 3.27 Aº distance and energy of -0.6 kcal/mol.



The interaction between carnosol and PBPs proteins:

The amino acid SER 392 (H-donor) with 2.61 Aº distance and energy of -2.1 kcal/mol.

The amino acid GLU 623 (H-acceptor) with 3.3 distance and energy of -1.6 kcal/mol



The interaction between rosmarinic acid and PBPs proteins:

The amino acid ASP 601 (H-donor) with 2.57 Aº distance and energy of -11.5 kcal/mol.

The amino acid SER 392 (Ionic) with 3.77 distance and energy of -1.0 kcal/mol

Figure 3: 2D diagram illustrate theoretical interaction from docking software between selected phenolic acids in rosemary extract and PBPs protein

Antibacterial and Antifungal activity of rosemary extract: Inhibition zone of rosemary extract against bacterial and fungal isolate illustrated in table 3, and Figure 2 showed the antibacterial and antifungal activity.

Also, table 4 illustrates the minimum inhibitory concentration for rosemary extract against bacterial and fungal isolates.

The antibacterial activity of rosemary has been determined in various assay types such as MIC, many studies demonstrate the antibacterial activity of rosemary extract is due to the presence of phenolic acids such as rosmarinic acid, carnosol, and carnosic acid, this compound interacts with the cell membrane, causing changes in genetic material and nutrients also produced an interaction with the membrane of proteins that produced the loss of membrane functionality and its structure (Nieto et al., 2018). Antifungal activity of rosemary extract is a moot case subject and the researches illustrate that phenolic acids affect the cell membrane of Gram-positive and Gram-negative bacteria leading to a change in cell surface hydrophobicity and charge, ultimately causing leakage of

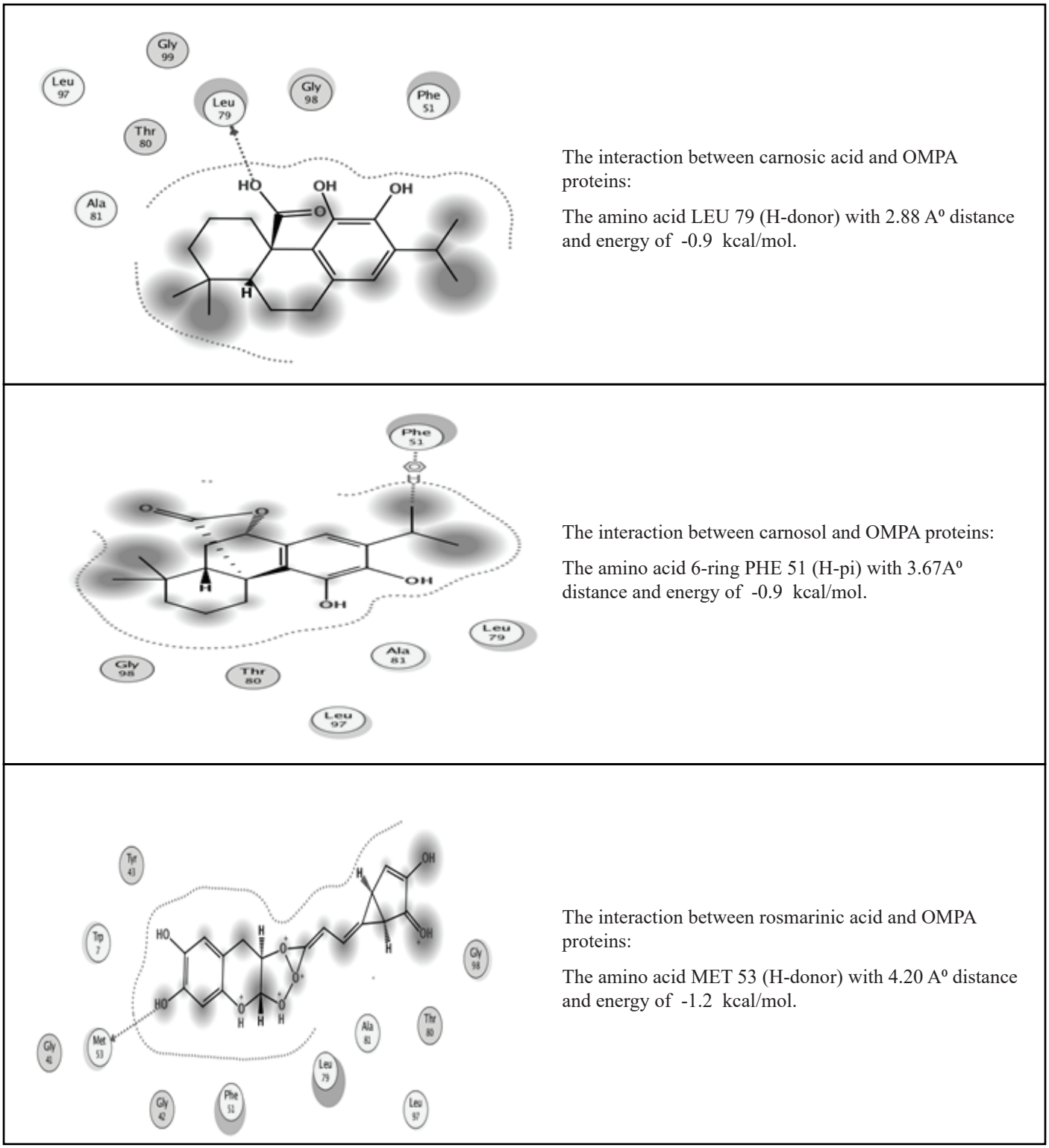
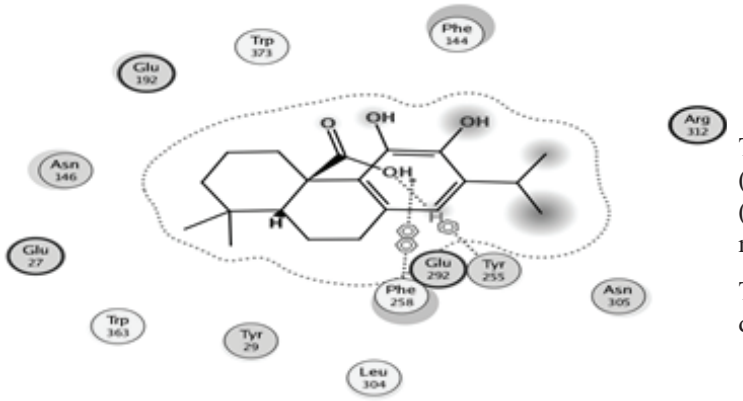


Figure 4: 2D diagram illustrate theoretical interaction from docking software between selected phenolic acids in rosemary extract and OMPA protein

cytoplasmic content, similar effect has been suggested for the phenolic acids on Candida cytoplasmatic membrane (Teodoro et al., 2015). The effect on other fungi varies between phenolic acid type and concentration but the mechanism of action is due to Phenolics interfere with the integrity of cell membranes or inhibit the germination of spores (Ejechi et al., 1999). These results make rosemary a good replacement for more toxic artificial food additives as concern over these potentially harmful additives has grown, so has the demand for natural preservatives such as rosemary 2017.

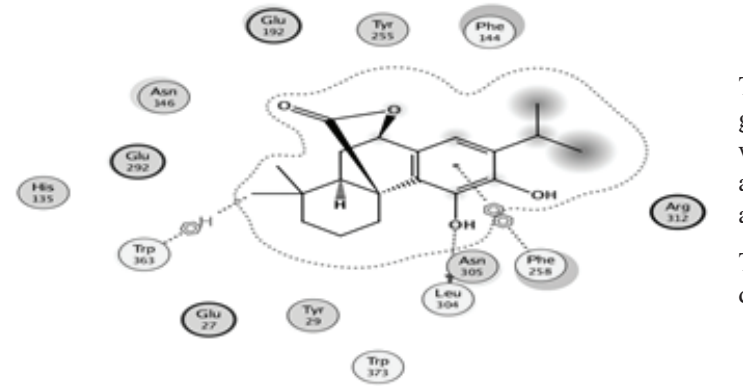
Molecular docking study to explain the antibacterial and antifungal activity of phenolic acids in rosemary extract

From different poses produced by MOE software, we chose the best conformation according to the good binding energy score and suitable interaction with the selected protein active site. For docking study we select the most effective phenolic acid as an antimicrobial agent,



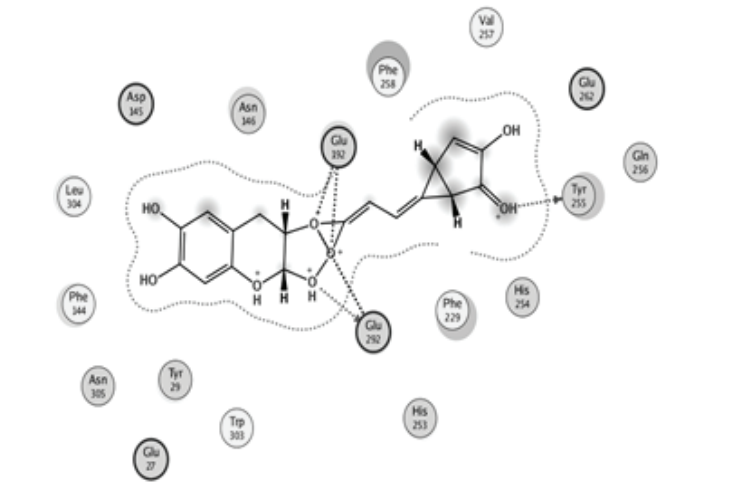
The interaction between carnosic acid and EXO-beta-(1,3)-glucanase protein: The amino acid 6-ring TYR 255 (H-pi) with 3.36 Aº distance and energy of -1.2 kcal/mol.

The amino acid 6-ring PHE 258 (H-pi) with 3.94 Aº distance and energy of -0.0 kcal/mol.



The interaction between carnosol and EXO-beta-(1,3)-glucanase protein: The amino acid 6-ring TRP 363 (H-pi) with 4.52Aº distance and energy of -0.6 kcal/mol. The amino acid 6-ring PHE 258 (pi-pi) with 3.94 Aº distance and energy of -0.0 kcal/mol.

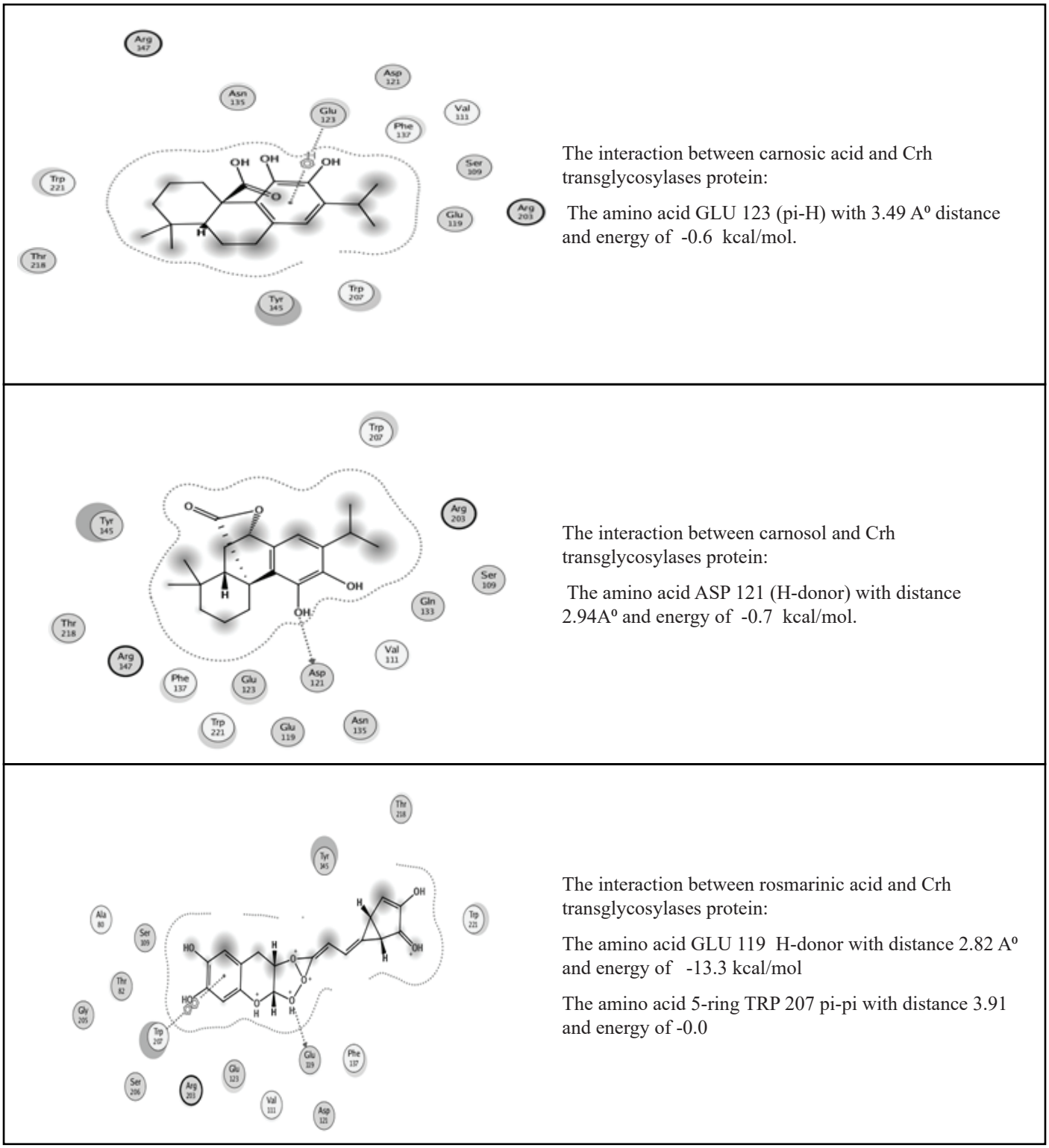
The amino acid LEU 304 (H-donor) with 2.82 Aº distance and energy of -1.7 kcal/mol.



The interaction between rosmarinic acid and EXO-beta-(1,3)-glucanase protein: The amino acid GLU292 H-donor with distance 3.03 Aº and energy of -15.7 kcal/mol, TYR 255 H-donor with distance 2.81 Aº and energy of -6.1 kcal/mol, GLU192(ionic) with distance 2.57 Aº and energy of -8.1 kcal/mol, GLU 192(ionic) with distance 2.75 and energy of -6.4 kcal/mol, GLU 292(ionic) with distance 3.93 Aº and energy of -0.7kcal/mol, GLU 292 (ionic) with distance 3.03 Aº and energy of -4.2 kcal/mol, GLU192(ionic) with distance 3.50 Aº and energy of -1.9kcal/mol, GLU 192 (ionic) 2.76 Aº and energy of -6.3kcal/mol, GLU 292 (ionic) with distance 2.92 Aº and energy of -5.0 kcal/mol

Figure 5: 2D diagram illustrate theoretical interaction from docking software between selected phenolic acids in rosemary extract and EXO-beta-(1,3)-glucanase protein

rosmarinic acids which is effective against many types of bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) which is resistant to all kinds of β-lactams, threatens even the most potent antibiotics (Ekambaram et al., 2016). In addition to carnosic acid and carnosol which are demonstrated by many studies that the antimicrobial activity against oral pathogens of *R. officinalis* leaf extract may be mainly attributed to the presence of these phenolic acids (Bernardes et al., 20100). To explain the antibacterial activity of selected phenolic acids we chose two bacterial proteins in the cell wall which abundant in gram-positive and gram-negative bacteria, Penicillin-Binding-Proteins (PBPs) which catalyze the polymerization of the glycan strand (transglycosylation) and the cross-linking between glycan chains (transpeptidation), some PBPs can hydrolyze the last D-alanine of stem pentapeptides (DD-carboxypeptidation) or hydrolyze the peptide bond connecting two glycan strands (endopeptidation) (Sauvage et al., 2008). The other protein is Outer membrane protein A (OMPA), the function of OmpA is thought to contribute to the structural integrity of the outer membrane along with murein lipoprotein and peptidoglycan-associated



The interaction between carnosic acid and Crh transglycosylases protein:

The amino acid GLU 123 (pi-H) with 3.49 Aº distance and energy of -0.6 kcal/mol.

The interaction between carnosol and Crh transglycosylases protein:

The amino acid ASP 121 (H-donor) with distance 2.94Aº and energy of -0.7 kcal/mol.

The interaction between rosmarinic acid and Crh transglycosylases protein:

The amino acid GLU 119 H-donor with distance 2.82 Aº and energy of -13.3 kcal/mol

The amino acid 5-ring TRP 207 pi-pi with distance 3.91 and energy of -0.0

Figure 5: 2D diagram illustrate theoretical interaction from docking software between selected phenolic acids in rosemary extract and Crh transglycosylases protein

lipoprotein (Wang 2002). Each selected phenolic acids have a good interaction with (PBPs) and (OMPA) proteins which explain the biological activity of rosemary extract in Figure 3,4 and Table 5. To demonstrate the antifungal activity we chose two fungal proteins EXO-beta-(1,3)-glucanase, beta-1,3-glucanases are of great importance, since the major structural component of the fruiting body cell wall is a beta-1,3-glucan, beta-glucanases are believed to play significant roles in the autolysis of cell-wall involved in morphogenesis, the metabolism of cell-wall components, and the maintenance of an energy source for cell survival under conditions of nutritional depletion (Fukuda et al., 2008). The other fungal protein is Crh transglycosylases which responsible for Covalent cross-links between chitin and glucan at the fungal cell wall (Blanco et al., 2015). Each selected gives a good interaction with these proteins which gives the antifungal activity of the rosemary extract Figure 5,6 and Table 6.

CONCLUSION

Rosmarinus officinalis L. form a good source for many phenolic acids; in this research the most popular

(rosmarinic acid, carnosic acid, and carnosol) were chosen because these phenolic acids have good antioxidant activity and attribute to biological activity against bacteria and fungi and this approved in docking study which demonstrates a good binding energy score with bacterial and fungal proteins.

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