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Antibacterial, Antifungal, Antioxidant, and Docking Studies of Potential Dinaphthodiospyrols from *Diospyros lotus* Linn Roots

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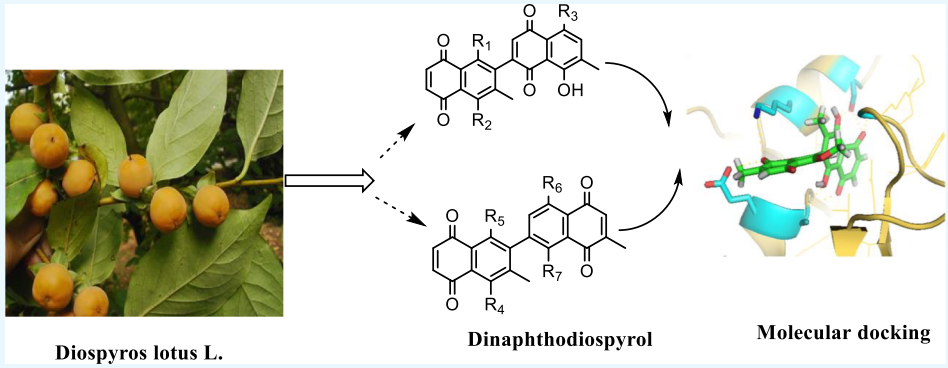

ABSTRACT: The main aims of this investigation were the isolation of dimeric naphthoquinones, a new class of dinaphthodiospyrols (**1**−**7**), from chloroform fractions and screening them for antibacterial, antifungal, and antioxidant potential. The susceptibility of the isolated compounds, namely, dinaphthodiospyrol A (**1**), dinaphthodiospyrol B (**2**), dinaphthodiospyrol C (**3**), dinaphthodiospyrol D (**4**), dinaphthodiospyrol E (**5**), dinaphthodiospyrol F (**6**), and dinaphthodiospyrol G (**7**) was assessed for antibacterial potential using well diffusion methods. The isolated compounds showed excellent antibacterial activity against selected bacterial strains, including Gram-positive *Bacillus subtilis*, *Streptococcus epidermis*, and *Bacillus subtilis*, and Gram-negative bacteria *Klebsiella pneumonia* with the zones of inhibition 6 to 26 nm. The standard drug Imipenem showed a maximum inhibitory zone 30 to 35 nm. Similarly, the isolated compounds were screened for antifungal properties, which showed an excellent reduction in the growth of selected fungal strain including *Candida albicans*, *Aspergillus flavus*, *Fusarium solani*, *Trichyton logifusus*, *Microsporum canis*, *and Candida glabrata*. Among all the screened compounds, **7** exhibited good activity (30−49 mm), followed by compounds **5** and **6**, (35−46 mm), while compounds **1**−**4** showed a moderate effect (8−28 mm) against the selected fungal strain against miconazole which showed potent effects (101−110.98 mm). The isolated compounds were also screened for 1, 1-diphenyl-2-picrylhydrazyl (DPPH) activity. In vitro-based free radical was employed using ascorbic acid as a standard antioxidant. The tested compounds (**1**−**7**) exhibited significant antioxidant activity in a concentration-dependent manner. The dinaphthodiospyrol **7** exhibited 97.32% scavenging activity, followed by dinaphthodiospyrol **6**, 92.01%, and compounds **5** and **4** with 89.90 and 88.43% scavenging activity at 100 μg/mL, respectively; ascorbic acid showed 96.45% scavenging effect. Furthermore, docking analysis was performed to know the exact binding mode of the tetra-substituted derivatives of dinaphthodiospyrols to the selected target proteins. From the docking analysis, it was found that the docking results are well correlated with the experimental observations. In conclusion, the dinaphthodiospyrols exhibited excellent antibacterial, antifungal, and free radical scavenging potential.

1. INTRODUCTION

Traditional medicines prepared from plants have documented to be clinically more effective and less toxic as compared to synthetic drugs. Therefore, traditional medicines are considered to be safe.[1,2] Plant extracts are the primary source of medicines for new drug discovery. There are a variety of bioactive compounds in plant extracts which largely depend on the type of solvent and the procedure of extraction.[1,3] For this purpose, a variety of solvents are used in order to purify a specific class of compounds. The commonly used organic solvent is *n*-hexane that is used for the extraction of nonpolar compounds, while methanol, ethanol, ethyl acetate, and water are used for the extraction of polar compounds.[4] Phytochemicals (natural products) are bioactive chemicals of plant origin. Secondary metabolites are naturally synthesized using different parts of the plants, including roots, stem, barks, leaves, flowers,

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Table 1. Antibacterial Activity of Compounds (1−7) Isolated from *D. lotus*[a]

	zone of inhibition (mm)								
bacterial strain	control	1	2	3	4	5	6	7	imipenem
E. coli	0								30.22 ± 0.07
K. pneumonia	0	06 ± 0.00	08 ± 0.12	16 ± 0.34	17 ± 0.06	18 ± 0.76	22 ± 0.06	20 ± 0.40	29.09 ± 0.04
S. aureus	0	12 ± 0.02	14 ± 0.14	16 ± 0.20	18 ± 0.07	16 ± 0.6	16 ± 0.012	14 ± 0.55	28.98 ± 0.70
S. epidermis	0	08 ± 0.03	10 ± 0.09	18 ± 0.33	19 ± 0.23	20 ± 0.08	24 ± 0.08	22 ± 0.64	30.21 ± 0.75
B. subtilis	0	14 ± 0.17	16 ± 0.08	20 ± 0.44	21 ± 0.28	22 ± 0.11	26 ± 0.02	24 ± 0.42	35.05 ± 0.56

[a]Data are represented as mean ± SEM of three different sets of individual experiments in each column.

Table 2. Antimicrobial Activity (MIC; μg/Ml) of Compounds (1−7) Isolated from *D. lotus*[a]

	MIC; μg/ml							
bacterial strain	control	1	2	3	4	5	6	7	imipenem
E. coli									0.18 ± 0.04
K. pneumonia				62 ± 0.09	66 ± 0.65	59 ± 0.65	50 ± 0.60	55 ± 0.55	0.20 ± 0.06
S. aureus				64 ± 0.07	64 ± 0.41	82 ± 0.45	83 ± 0.43	65 ± 0.44	0.22 ± 0.00
S. epidermis				60 ± 0.23	59 ± 0.43	60 ± 0.34	44 ± 0.70	45 ± 0.60	0.19 ± 0.02
B. subtilis			90 ± 65	54 ± 0.33	50 ± 0.12	75 ± 0.30	25 ± 0.60	40 ± 0.34	0.21 ± 0.08

[a]Data are represented as mean ± SEM of three different sets of individual experiments in each column.

Table 3. . Antifungal Effect of Compounds (1−7) Isolated from *Diospyros lotus*[a]

	% zone of inhibition							
fungal strain	l	2	3	4	5	6	7	miconazole; MIC (μg/ml)
C. albicans	20.98 ± 1.09	22.80 ± 1.08	26.98 ± 1.00	28.31 ± 1.67	40.32 ± 1.22	44.22 ± 1.20	45.99 ± 1.29	110.98
A. flavus								20.40
F. solani	8.23 ± 1.56	12.34 ± 1.09	10.76 ± 1.98	16.23 ± 1.67	46.0 ± 1.87	40.0 ± 1.60	42.0 ± 1.68	108.98
T. logifusus	10.22 ± 1.01	20.90 ± 1.20	22.98 ± 1.78	25.11 ± 1.02	35.09 ± 1.66	38.09 ± 1.60	30.09 ± 1.61	106.76
M. canis	8.21 ± 1.23	10.10 ± 1.30	12.09 ± 1.07	18.00 ± 1.07	42.22 ± 1.03	40.29 ± 1.05	45.29 ± 1.09	104.22
C. glabrata	10.23 ± 1.56	18.90 ± 1.87	20.98 ± 1.06	22.08 ± 1.76	40.01 ± 1.00	46.09 ± 1.04	49.09 ± 1.09	101.23

[a]Data are represented as mean ± SEM of three different sets of individual experiments in each column.

and seeds.[1,4] Phytochemicals have been identified as the basis for traditional medicine used throughout the globe in the past and now. Medicinal plants are commonly screened for various secondary metabolites that are present before bulk extraction. The identification of various classes of active phytochemicals leads to its detailed isolation, purification, and structure elucidation of simple and complex natural products, which can be used as the basis for new pharmaceutical products.[1,4]

Free radicals are reactive species generated in our body as a result of several biochemical reactions. These free radicals are harmful and help in the development of disease conditions and cause inflammation, cancer aging, and many others.[5] Therefore, their scavenging is necessary for a healthy body. Human body has its own free radical scavengers like protein. To counteract the free radicals, some supplementary antioxidants are used; therefore, the secondary metabolites play an important role in the countering of free radicals.[5] It has been found that *Diospyros lotus* contains some of the bioactive secondary metabolites that act as antioxidants.[6] The use of medicinal plants against bacteria and fungi is trending because of its effectiveness. Therefore, researchers are trying to investigate the medicinal plants which are efficient against microbes.[6]

Diospyros lotus Linn is an important medicinal plant that belongs to the family Ebenaceae.[7] It is a deciduous tree found in Asia, China, and Japan. It is found in semishade places, and its height reaches 9 m.[8] *Diospyros lotus* has been cultivated for its edible fruits in many countries.[8] Their fruits are globose in shape, and they are bluish-black or yellow in color. The fruits of *Diospyros lotus* are used as a folk medicine for the treatment of constipation, as a sedative, as an antidiabetic, antitumor, and laxative agent, as well as a febrifuge and nutritive.[9] The fruits of *Diospyros lotus* are also used as an antiseptic, antidiabetic, antitumor, and antihypertensive agent. It has been found that its fruits are also effective against dry cough and diarrhea.[10] Its fruits are also used for the treatment of dry cough and hypertension.[10] Phytochemical investigation of *Diospyros lotus* indicated the presence of dimeric naphthoquinones, steroids, naphthalene derivatives, and lupine triterpenes.[11−13] The phytochemical studies of the fruits indicated the presence of fatty acids, nonvolatile acids, phenolic groups, and sugars.[14] Phytochemicals such as diospyrin and 8-hydroxdiospyrin have been reported for their excellent analgesic, sedative, and anti-inflammatory effects.[15,16] The recently reported dinaphthodiospyrol (A−H) has been reported for analgesic, sedative, muscle relaxation, antiulcer and anticancer properties.[17−20] In this work, we try to understand and determine the antioxidant and antimicrobial activities of the dimeric naphthoquinones (**1**−**7**) purified from *Diospyros lotus* against infectious diseases.

2. RESULTS

2.1. Effect of Dinaphthodiospyrols (1−7) against Selected Bacteria. The isolated dinaphthodiospyrols (**1**−**7**) from *Diospyros lotus* was also assessed for antibacterial sensitivity against the selected Gram-positive and Gram-negative bacterial strain (Tables 1 and 2). The dinaphthodio-

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Table 4. . Antioxidant Activity of Compounds (1−7) Isolated from *Diospyros lotus*[a]

	% DPPH							
conc. (μg/mL)	1	2	3	4	5	6	7	ascorbic acid
5	35.43 ± 1.43	33.40 ± 1.49	36.55 ± 1.05	44.09 ± 1.05	46.32 ± 1.32	49.22 ± 1.01	51.09 ± 1.30	90.23 ± 1.00
10	41.00 ± 1.50	39.09 ± 1.23	44.01 ± 1.09	52.08 ± 1.23	56.44 ± 1.01	59.43 ± 1.03	62.87 ± 1.43	91.10 ± .1.06
20	48.23 ± 1.36	46.98 ± 1.00	49.33 ± 1.04	60.43 ± 1.54	63.98 ± 1.34	68.11 ± 1.23	70.09 ± 1.05	91.80 ± 1.20
30	53.98 ± 1.98	51.91 ± 1.90	55.90 ± 1.34	70.66 ± 1.43	73.23 ± 1.23	77.32 ± 1.43	79.23 ± 1.76	91.83 ± 1.50
40	60.45 ± 1.97	58.44 ± 1.05	62.01 ± 1.23	75.23 ± 1.43	77.09 ± 1.54	80.09 ± 1.09	83.09 ± 1.98	91.87 ± 1.98
60	69.22 ± 1.23	67.20 ± 1.98	70.88 ± 1.90	78.09 ± 1.00	81.12 ± 1.75	86.23 ± 1.32	88.87 ± 1.06	92.99 ± 1.23
80	74.43 ± 1.40	72.46 ± 1.43	75.09 ± 1.66	81.06 ± 1.02	84.10 ± 1.32	90.02 ± 1.09	93.98 ± 1.03	93.32 ± 1.07
100	79.99 ± 1.09	77.09 ± 1.22	82.98 ± 1.66	88.43 ± 1.02	89.90 ± 1.00	92.01 ± 1.32	97.32 ± 1.43	96.45 ± 1.09

[a]Data are represented as mean ± SEM of three different sets of individual experiments in each column.

spyrols (**1**−**7**) showed excellent activity against the selected bacterial strain, including Gram-positive *Bacillus subtilis*, *Streptococcus epidermis*, and *Bacillus subtilis*, and Gram-negative bacteria *Klebsiella pneumonia* with a zone of inhibition 6 to 26 nm. Among the tested strain, the compound did not show any susceptibility against *Escherichia coli*.

2.2. Effect of Dinaphthodiospyrols (1−7) against Selected Fungi. The purified dinaphthodiospyrols (**1**−**7**) from *Diospyros lotus* was also evaluated for antifungal sensitivity against the selected fungal strain (Table 3). The results showed that the tested compounds showed an excellent reduction in the growth of the selected fugal strain including *Candida albicans*, *Aspergillus flavus*, *Fusarium solani*, *Trichyton logifusus*, *Microsporum canis*, and *Candida glabrata*. Among all the screened compounds, **7** exhibited good activity (30−49 mm), followed by compounds **5** and **6**, (35−46 mm), while compounds **1**−**4** showed moderate affected (8−28 mm) against the selected fungal strain.

2.3. Antioxidant Effect Dinaphthodiospyrols (1−7). The antioxidant potential of isolated dinaphthodiospyrols (**1**−**7**) from *Diospyros lotus* is listed in Table 4. The isolated compound was screened at accumulating concentrations such as 5, 10, 30, 20, 60, 80, and 100 μg/mL (Table 4). Among the tested dinaphthodiospyrols (**1**−**7**), the maximum antioxidant effect was demonstrated by compounds **7** (97.32) and **6** (92.01), followed by compounds **5** and **4** with 89.90 and 88.43% scavenging, respectively, at higher dose (100 μg/mL) (Table 4).

2.4. Molecular Docking. Molecular docking is a computational technique used to find out the interaction of ligands within the active site of target proteins.[21] To computationally identify target proteins for antifungal and antibacterial activity of newly synthesized, tetra-substituted dinaphthodiospyrols, seven different drug target proteins, that is, dihydrofolate reductase (DHFR) (PDB ID 4HOF), secreted aspartic protease (PDB ID 3Q70), and *N*-myristoyl transferase (PDB ID 1IYL) from *Candida albicans* were selected as the antifungal targets, whereas drug targets dihydrofolate reductase (PDB ID 3FYV), gyrase B (PDB ID 4URM), and sortase A (PDB ID 2MLM) from *Staphylococcus aureus* and rhomboid protease (PDB ID 3ZMI) from *Escherichia coli* were selected as antibacterial targets. The three-dimensional structures of all the target proteins were downloaded from the Protein Data Bank. All the isolated compounds were docked into the active sites of the selected target proteins. The docking results showed that all these compounds showed good interactions with two target proteins, that is, dihydrofolate reductase from *C. albicans* and dihydrofolate reductase from *Staphylococcus aureus*, while they showed poor interactions with the rest of the target proteins. Therefore, the binding modes of these compounds within the active sites of the dihydrofolate reductase fungal protein (PDB ID 4HOF) and dihydrofolate reductase bacterial protein (PDB ID 3FYV) were studied in more detail. The docking results showed that all the compounds fit well in the binding pockets of dihydrofolate reductase (fungal) as well as in the bacterial dihydrofolate reductase target proteins. Among them, compound **7** showed good interactions with the dihydrofolate reductase (fungal) (PDB ID 4HOF), dihydrofolate reductase (*Staphylococcus aureus*) (PDB ID 3FYV), and with *C. albicans* *N*-myristoyl transferase (PDB ID 1iyl). The docking conformation of compound **7** showed that this compound established seven hydrogen bonds and several hydrophobic interactions with active site residues of the target protein (Figure 1). The hydrogen bonds were observed between the

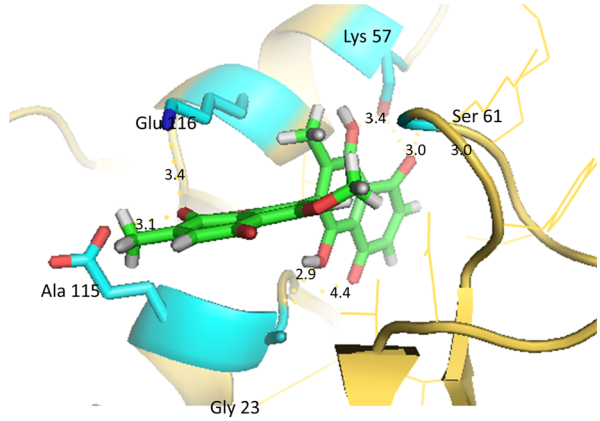

Figure 1. Molecular docking conformation of compound **7** in the active site of *C. albicans* dihydrofolate reductase protein (PDB ID 4HOF) showing hydrogen bonding, and hydrophobic and van der Waals interactions.

compound and active site residues Glu 116, Ala 115, Lys 57, Ser 61, and Gly 23 of the target protein, dihydrofolate reductase (Figure 1). Similarly, this compound also showed good interactions with bacterial dihydrofolate reductase (*Staphylococcus aureus*). The docking conformation of compound **7** in the active site of bacterial dihydrofolate reductase showed that this compound formed hydrogen bonds with active site residues, Ile 14, Asp 120, Asn 18, Lys 45, and Thr46 (Figure 2). The fungal protein N-myristoyl transferase (PDB ID 1IYL) from *Candida albicans* also showed somewhat good interactions with compound **7** (Figure 3). The docking conformation showed that this compound formed two hydrogen bonds with active site residues His 227 and Asn

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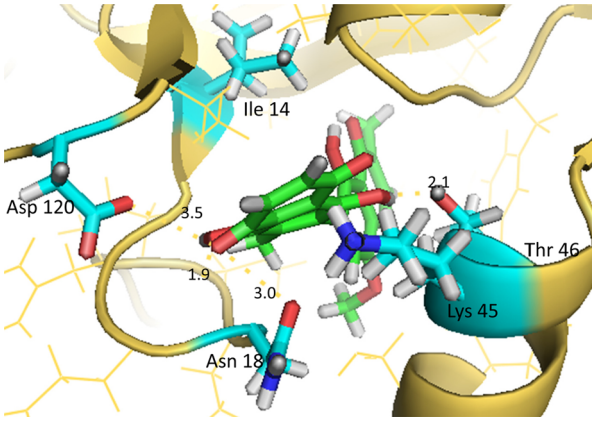

Figure 2. Molecular docking conformation of compound **7** in the active site of *Staphylococcus aureus* dihydrofolate reductase (PDB ID 3FYV) showing hydrogen bonding, and hydrophobic and van der Waals interactions.

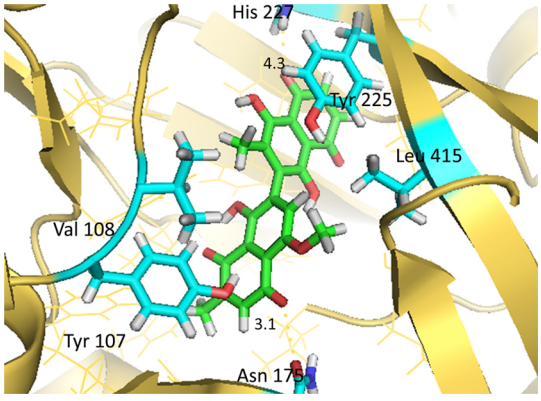

Figure 3. Molecular docking conformation of compound **7** in the active site of *C. albicans N-myristoyl transferase* protein (PDB ID 1iyl) showing hydrogen bonding, and hydrophobic and van der Waals interactions.

175, while hydrophobic interactions with active site residues Tyr 225, Tyr 107, and Leu 415 were observed (Figure 3). The docking results of compounds **5** and **6** also showed good interactions with target proteins, especially with the fungal dihydrofolate reductase. In case of compound **6**, the docking results showed that this compound formed hydrogen bonds with active site residues, Thr 147, Ile 119, Tyr 118, Thr 58, and Ser 61 (Figure 4). Similarly, compound **5** established seven hydrogen bonds with important active site residues (Gly23, Lys 57, Ser 61, Ala 115, and Thr 147) and a number of hydrophobic interactions with the active site residues of dihydrofolate reductase (Figure 5).

The other compounds (compounds **1**−**4**) showed poor or no interactions with the binding residues of target proteins. The poor interaction of these compounds with the target proteins might be due to the presence of different bulky groups attached at different sites of these compounds. As these bulky groups are absent in compounds **5**−**7**, the different orientations of attachment of different groups to the same chemistries alter the binding capability of these compounds. This might be one of the reasons that these compounds showed biological activities and different interactions with the target proteins.

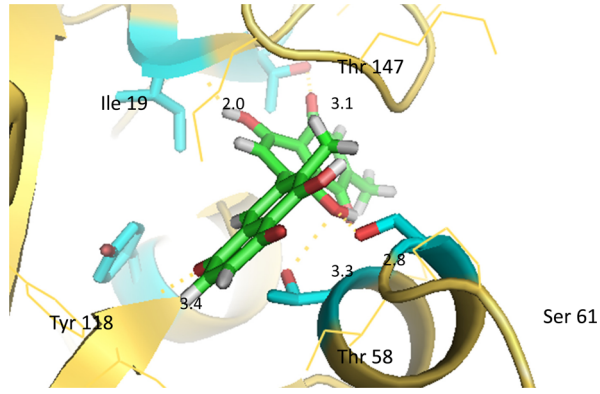

Figure 4. . Molecular docking conformation of compound **6** in the active site of *C. albicans* dihydrofolate reductase (PDB ID 4hof) showing different intermolecular interactions.

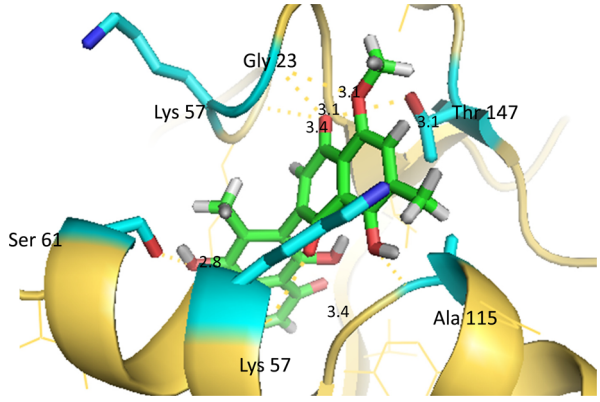

Figure 5. Molecular docking conformation of compound **5** in the active site of dihydrofolate reductase (PDB ID 4hof) showing different intermolecular interactions.

3. DISCUSSION

Dimeric naphthoquinones, namely, dinaphthodiospyrols (**1**−**7**) was isolated as a red amorphous powder from the chloroform fractions of *Diospyros lotus*. The chemicals structures of isolated dinaphthodiospyrols (**1**−**7**) were identified by our research group using advanced spectroscopic techniques including electron ionization mass spectroscopy (EI−MS), H-NMR, C-NMR, heteronuclear multiple bond correlation (HMBC), and heteronuclear single quantum spectroscopy (HSQC).[17,18]

The stoppage of the spoilage of food and the food poising of pathogens is normally attained with the use of chemical preservatives which exhibits side effects due to chemical residues in food and causes human health hazards due to the application of chemicals, and gains microbial resistance to the usage of chemicals.[22] Because of such concerns, it is essential and better to find healthier, safer, potentially effective, as well as a natural alternative preservative.[22] With this text, medicinal plant-derived compounds have been used to regulate the poisoning of food stuff as well as preserve food. The natural products derived from medicinal plants have been used widely, both in present as well as in the past, for the cure of various diseases, and these phytochemicals provide a very excellent template for the discovery of novel drugs.[22] The current study deals with the isolation of dinaphthodiospyrols (**1**−**7**) from chloroform fractions and their in vitro antibacterial, antifungal, and antioxidant potential.

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It has been identified across the globe that the irrational uses of antibiotics are accountable for the proliferation of pathogenic bacteria with developing resistance to different classes of antibiotics.[23] Precocious, this has controlled the advanced damage in the therapeutic efficiency of antibiotics at a degree that differs with the complexity of essential mutations, the affluence of clonal spread, or the rate of horizontal gene transfer. The prevalence of infection because of multidrug-resistant pathogens has shown an intense increase throughout the world. Several new antibiotics have been discovered to combat infectious diseases, but multidrug resistance genes have been seen rapidly among bacterial pathogens to develop resistance to antibiotics.[24]

The isolated dinaphthodiospyrols (**1**−**7**) showed an excellent antibacterial effect against the selected Gram-positive and Gram-negative bacterial strain (Tables 1 and 2). The dinaphthodiospyrols (**1**−**7**) showed an excellent activity against the selected bacterial strain including Gram-positive *Bacillus subtilis*, *Streptococcus epidermis*, and *Bacillus subtilis*, and Gram-negative bacteria *Klebsiella pneumonia* with a zone of inhibition 6 to 26 nm. Among tested strain, the compounds **1**−**7** did not show any susceptibility against *Escherichia coli*. Correspondingly, with an extended growth in the number of fungi-infected patients around the world, there has been observed deviation in the fungal pathogen. The fungus normally cause the infection in the patient with bone narrow, surgery, cancer, solid organ transplant receiver, and additional immune block and extremely affected patients infected with human immunodeficiency virus (HIV).[23] The dinaphthodiospyrols (**1**−**7**) was assessed for fungal potential against selected fungal strain including *Candida albicans*, *Aspergillus flavus*, *Fusarium solani*, *Trichyton logifusus*, *Microsporum canis*, and *Candida glabrata*. Among all the screened compounds, **7** exhibited good activity (30−49 mm), followed by compounds **5** and **6**, (35−46 mm), while compounds **1**−**4** showed a moderate effect (8−28 mm) against the selected fungal strain.

Radicals produced in the body are involved in various processes which causes mutations. Additionally, free radical reactions are also involved in many chronic diseases such as heart disease, cataracts, cancer hypertension, rheumatism, and many others which affect the lifestyle of human beings.[25,26] Based on the mode of action, antioxidants are classified as free radical terminators, chelators of metals ions which are involved in catalyzing the scavenging of oxygen, or/as well as lipid oxidation which reacts with oxygen-closed systems.[27] The natural products having antioxidant capacity or antioxidants are free radical scavengers that provide protection to the human body against radicals and inhibit different oxidizing chain reactions.[28] When various constituents exist at a lower concentration in the human body, they prevent the oxidation of an oxidizable substrate. The antioxidant plays a key role in suspending the development of chronic disorders including cancer, atherosclerosis, and inflammatory and cardiovascular disease. Antioxidants derived from medicinal plants play a principal part in serving endogenous antioxidants to neutralize oxidative stress.[27−30] Numerous clinical, epidemiological, as well as experimental data recommend that medicinal plants based on natural antioxidants have valuable effects on the inhibition of chronic diseases. Because of less toxic effects and excellent antioxidant potential, there is keen interest in screening the role of active phytochemicals from plants in reducing the threat of the mentioned diseases.[27] Different procedures are employed for the identification of antioxidant activity, but DPPH has achieved significant importance currently because of easiness as well as simplicity. The results of our findings indicated the outdated potential of dinaphthodiospyrols (**1**−**7**) in a concentration-dependent manner. The compounds (**5**−**7**) exhibited a maximum scavenging effect of 97.32, 92.01, and 88.43%, respectively, at 100 μg/mL (Table 4). The results of our study indicated that dinaphthodiospyrols (**1**−**7**) interfered with the activity of stable free-radical DPPH.

The docking results of all the seven compounds showed that the most active compound (compound **7**) showed good interactions with fungal and bacterial target protein dihydrofolate reductase. This compound also showed somewhat good interactions with another fungal protein *N*-myristoyl transferase. Furthermore, compounds **5** and **6** also showed good interactions with target protein fungal dihydrofolate reductase. The good interactions of compounds **7**, **5**, and **6** might be due to the formation of strong hydrogen-bonding network with the active site residues of target proteins. Whereas the poor interaction of compounds (compounds **1**−**4**) might be due to the presence of different bulky groups attached at different sites of these compounds. As these bulky groups are absent in compounds **5**−**7**, the different orientations of the attachment of different groups to the same chemistries alter the binding capability of these compounds. This might be one the reasons that these compounds showed biological activities and different interactions with the target proteins.

Based on the results obtained in the present study, we recommended *D. lotus* for further isolation of new and novel compounds and synthesis of its derivatization to discover potent antimicrobial and antioxidant compounds with clinical applications.

4. MATERIALS AND METHODS

4.1. Plant Material Collection. The fresh roots of *Diospyros lotus* were obtained from the mountain areas of Razagram, Dir, KPK, Pakistan. The plant specimen was identified by Professor Dr. Abdur Rahid, Department of Botany, University of Peshawar, KPK, Pakistan. The plant specimen no (Bot.20036(PUP) was kept in the herbarium of the Department of Botany, University of Peshawar, KPK, Pakistan.

4.2. Extraction, Fractionation, and Isolation. The plant materials (roots) were dried in shade at room temperature. The dried root materials were chopped with the help of a cutter and then subjected to cold extraction. The maximum numbers of naphthoquinones were soluble in chloroform. For the purpose of naphthoquinones extraction with a high degree of accuracy, chloroform was used for extraction. The shade-dried plant material (10 kg) was subjected to cold extraction with chloroform for 15 days. After 15 days, the obtained extract was filtrated and then concentrated by evaporating the solvent with the help of a rotary evaporator at a reduced pressure and low temperature (50 °C). Extraction was repeated three times until the maximum number of extraction was completed, which afforded 180 g of deep reddish residue. The obtained crude chloroform extract was defatted with *n*-hexane using the soxhlet apparatus to remove color and dye, which yield 144 g of chloroform extract. Among the defatted extract, 23 g was subjected to normal-phase column chromatography on Merck silica gel 60 (0.063−0.200 mm). Elution was started from *n*-hexane, and gradually, the polarity was increased with ethyl acetate (100:0 → 0:100), which yield

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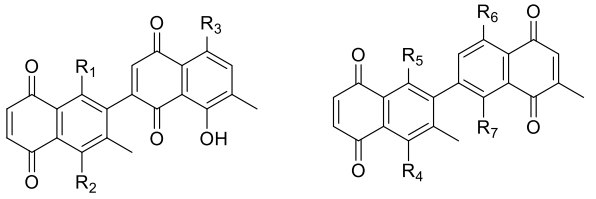
subfractions AF-1 to-130. Based on the thin-layer chromatography (TLC) profile, the subfraction AF-12 was subjected to the repeated chromatogram using *n*-hexane and ethyl acetate (11:89), which afforded compounds **1**−**5**. Similarly, the subfraction AF-20 and AF-21 were combined and submitted to repeated chromatography using *n*-hexane and ethyl acetate (13:8), which yielded compounds **6**−**9**. The purity of all the isolated compounds was checked by TLC. The chemical structures of isolated compounds (**1**−**7**; Figure 6; Scheme S1) were identified by comparing the physical and spectral data with the reported data.[17−19]



1: R_1=H; R_2=OCH_3; R_3=OH
2: R_1=OH; R_2=H; R_3=OH
3: R_1=R_2=H; R_3=OH
4: R_1=R_2=OCH_3; R_3=OH
5: R_1=R_2=OH; R_3=OCH_3
6: R_4=OH, R_5=H; R_6=R_7=OH
7: R_4=R_5=OH; R_6=OCH_3;R_7=OH

Figure 6. Chemical structures of compounds (**1**−**7**) isolated from *D. lotus*

4.3. Bacterial and Fungal Strains. Bacterial and fungal strains used in this study were obtained from the stock culture of the Department of Biological Sciences, King Abdulaziz University, Saudi Arabia. The bacterial strains include *E. coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Staphylococcus epidermis*, and *Bacillus subtilis* clinical isolates. Pathogenic fungi include *Candida albicans*, *Aspergillus flavus*, *Fusarium solani*, *Trichyton logifusus*, *Microsporum canis*, *and Candida glabrata* clinical isolates. The obtained organisms were maintained in Muller-Hinton Agar (MHA) in the refrigerator at 40 °C proceeding to subculture.

4.4. Antimicrobial Activity. The antibacterial activities of isolated compounds were obtained using a well diffusion procedure, as per our reported method.[20] The MHA medium was used for this experiment. The culture was prepared in triplicate, and incubation was achieved at 37 °C for 24−72 h. Then, the broth culture (0.6) of the tested microbe was positioned in a sterile Petri dish, and 20 mL of the sterile molten MHA was added. After the preparation of culture, the hole was bored with the help of a borer in the medium. Each compound (0.2 mL) was introduced to the well; similarly, the standard antibacterial drug (Imipenem) was also introduced in one well of each Petri dish. Incubation was performed for the first hour to make the diffusion of the antibacterial agent into the prepared medium possible. The incubation process was performed for 24 h at 37 °C after 24 h of incubation; the diameters of the inhibitory zone of each bacterial strain were measured in the plate in millimeters, which indicates that *D. lotus* exhibits significant antimicrobial activity.

4.5. 4.5. Antifungal Activity. The antifungal effect of isolated compounds from *Diospyros lotus* was assayed as per the standard method.[21] To check the fungal sensitivity, tube dilution was assessed to identify the fungal inhibition effect. The isolated compounds were dissolved in DMSO at 2 mg/mL to prepare the stock solution. Sabouraud dextrose agar (SDA) (4 mL) was put in each tube and autoclaved at 120 °C for 15 min. When this process was completed, it was allowed to cool at 15 °C. Then, the stock solution (66.6 mL) and SDA were mixed, which yielded the finial concentration of 2 mg/mL. The tubes were allowed to solidify in the slanted position at standard conditions. After that, every tube was incubated with the inoculum removed from the seven-days-old culture of the selected fungal strains. The antifungal effect of compounds and standard drugs against the selected fungal strain was determined after 7 days of incubation. The assay was performed in triplicate. Then, the results were analyzed for the visible growth of fungi, and the percentage effect was measured as per standard methods.

4.6. Antioxidant Activity. The antioxidant effect of isolated compounds from *Diospyros lotus* was determined, as per our reported methods,[22] using DPPH (diphenyl-1-picrylhydrazyl) using ultraviolet (UV) spectrophotometry at 517 nm. Each compound (2 mg) was dissolved in 100 mL of methanol to prepare a stock solution of each compound. Similarly, 1 mM solution of DPPH was prepared by dissolving 9.5 g of DPPH in 25 mL of methanol, as per our reported methods. Various concentrations such as 5, 10, 30, 20, 60, 80, and 100 μg/mL of each compound were prepared using the dilution formula. Now, 1 mL of DPPH solution was combined with 4 mL of the sample solution in methanol, comprising 5−10 μg/mL and control. All the samples were maintained in the dark for 30 min, and absorbance was recorded at 517 nm with the help of UV spectrophotometry. The decrease in the DPPH absorbance states an increase in the DPPH radical scavenging effect. The scavenging of free radicals by DPPH is defined as the percent radical scavenging activity. The percent antioxidant activity was calculated using the below formula.

$$\%\text{DPPH} = [(\text{A0})\text{control abs} - (\text{A1})\ \text{Sample Abs}] \times 100/(\text{A0})\text{control abs.}$$

A0 = control is the absorbance of the blank sample and A1 = sample is the absorption of the standard sample.

4.7. Statistical Analysis. The obtain results were represented as the mean ± SEM of three assays in each case with the help of Graphpad Prism 6 software.

4.8. Molecular Docking. Molecular docking is a computational technique used to find out the interactions of ligands within the active site of target proteins.[23] Molecular docking was performed to know the best compound among the tetra-substituted thiophene derivatives to block bacterial and fungal proteins. For this purpose, PDB structures of all the seven proteins, that is, dihydrofolate reductase (DHFR) (PDB ID 4HOF), secreted aspartic protease (PDB ID 3Q70), and N-myristoyl transferase (PDB ID 1IYL) from *Candida albicans* were selected as antifungal targets, whereas targets dihydrofolate reductase (PDB ID 3FYV), gyrase B (PDB ID 4URM), and sortase A (PDB ID 2MLM) from *Staphylococcus aureus* and rhomboid protease (PDB ID 3ZMI) from *Escherichia coli* were downloaded from the PDB by visiting www.rcsb.org; then, tetra-substituted thiophene derivatives were docked into the binding site of these proteins using the docking protocol implemented in Molecular Operating Environment (MOE).

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5. CONCLUSIONS

It is concluded that isolated dinaphthodiospyrols (**1**−**7**) exhibited profound antibacterial sensitivity against various Gram-positive *Bacillus subtilis, Streptococcus epidermis, and Bacillus subtilis*, and Gram-negative bacteria *Klebsiella pneumonia*. Similarly, the compounds showed excellent antifungal activity against the selected fungal strain and validated the folkloric usage of *D. lotus* in the treatment of infectious diseases. The isolated dinaphthodiospyrols (**1**−**7**) show promising antioxidant activity in a concentration-dependent manner. In conclusion, di-naphthodiospyrol exhibited excellent antibacterial, antifungal, and free radical scavenging potential. The results were also analyzed computationally using the molecular docking approach. From docking analysis, it was also found that among all the tested compounds, compound **7** exhibited the best antifungal and antibacterial activity. The docking results are correlated with the experimental results as the highly active compounds (compounds **5**, **6**, and **7**) showed good interactions with the active site residues of the target proteins as compared to poorly active compounds (compounds **1**−**4**). Thus, dinaphthodiospyrols should be strong candidates for more detailed studies to identify more effective lead phytochemicals exhibiting antimicrobial and antioxidant activities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c06297.

Extraction and isolation of dinaphthodiospyrols (1−7) from Diospyros lotus (PDF).

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Notes

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