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RESEARCH ARTICLE

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Chemical Characterization, Antioxidant, Antimicrobial, Cytotoxicity and *in Silico* Studies of Hexane Extract and Essential Oils from *Citrus limon* Leaves

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The present study investigates the chemical composition, antioxidant and antimicrobial bioactivities of essential oil and hexane extract from *Citrus limon* leaves. The isolation of essential oil was carried out using the Clevenger apparatus. The percentage yield of essential oil and hexane extract from *Citrus limon* leaves was 0.59 and 0.50%, respectively. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay highlighted that *Citrus limon* leaves essential oil (CLEO) and hexane extract exhibited the significant antioxidant potential of 69.64 and 67.55%, respectively, compared to the BHT standard. Similarly, a significant inhibition in linoleic acid peroxidation was recorded in both CLEO (81.93%) and hexane extract (50.34%). Characterization of chemical constituents in CLEO and extract was executed using GC/MS, where Limonene was detected as a major compound in CLEO (60.52%) and hexane extract (73.62%). The haemolytic activity ranged from 2.46 to 5.75% revealing negligible cytotoxicity of CLEO and hexane extract. *In silico* studies agree with the *in vitro* antimicrobial studies, where vinimalol, taraxasterol, and moretenol present in CLEO showed strong interactions/inhibition against dihydroorotase and DNA gyrase from *E. coli*, and the tyrosyl-tRNA synthetase and DNA gyrase from *S. aureus*. Based on the current data, it may be concluded that both CLEO and hexane extract possessed significant bioactivities, such as antimicrobial and antioxidant activity, with minimal cytotoxicity.

Keywords: molecular docking, DPPH, MIC, Zone of inhibition, bioactivities, haemolytic activity.

Introduction

Categorically, 400,000 species of plants on earth have been declared to have medicinal value and have the potential to cure various remediless diseases and can also be used in several cosmetic formulations and food recipes.[1] Therefore, researchers are interested in finding bioactive compounds for various applications. In this respect, myriad extraction protocols have been developed to isolate bioactive constituents from various plants.[2] Synthetic antioxidants such as butylated hydroxyanisol, butylated hydroxytoluene, and propyl gallate are used to preserve food as an antioxidant. The use of synthetic antioxidants has negative effects on human health and may cause cancer and other degenerative diseases.[3] The literature also shows that plant extracts showed a remarkable inhibitory effect against pathogenic bacterial strains with significant minimum inhibitory concentration.[4]

Terpenoids and aromatic compounds are found in essential oils, which have been investigated and separated by various researchers from different plants due to their special aroma and biological potential.[5] The basic structure of terpenes is the isoprene unit (2-methyl-1, 3-butadiene) and is represented by the general structural formula $(C_5H_8)_n$, where n is denoted by the number of joined isoprene units.[6] This forms the basis of terpenes classification, divided further into other classes such as monoterpenes, diterpenes, triterpenes, sesquiterpenes, tetraterpenes, and polyterpenes.[7]

The essential oils are present in the various parts of odoriferous plants, such as flowers, stems, buds, fruits, leaves, roots, and seeds.[8] The amount of essential oils in plants may vary depending on different factors such as age, harvesting regions, and stage of maturity.[9] The extraction technique employed also impacts the yield of oil production. Currently, the most widely used methods for the isolation of essential oil are hydrodistillation and steam distillation.[10]

The genus *Citrus* belonging to the *Rutaceae* family, comprises diversified varieties such as lemon, lime, orange, and grapefruits. These are cultivated in Pakistan, Egypt, China, United States, India, and Spain.[5] According to literature data, *Citrus* fruits exhibit certain anti-inflammatory/antioxidant activities and help in the reduced onset of coronary and cancer diseases that might be linked to the presence of a wider range of phytochemicals such as terpenoids, flavonoids, limonoids, and tannins. The parts of citrus fruits, such as leaves, peels, and seeds, are enriched with multiple bioactive compounds. The parts of citrus thus may be employed in the food, cosmetic and pharmaceutical industries. However, these agro wastes are discarded yearly without revalorizing them into valuable products such as essential oils.[1] The literature survey shows that essential oils have greater biological activities due to the synergistic or additive effects of their compounds.[11,12] Nowadays, modern bioinformatic tools and protocols, such as molecular modeling and docking, provide an understanding of how various ligands may interact with the target proteins at the molecular level.[13] In this research, molecular docking has helped us to elucidate the binding mechanism of essential oil phytoconstituents with key bacterial enzymes.[14] The presence of antioxidants in essential oils isolated from *Acca sellowiana* leaves and stems also suggest their medicinal potential for anti-aging.[15]

Due to the climatic catastrophe being faced by Earth due to global warming, there is an enormous emphasis on circular economy and recycling of wastes by converting them into feedstock or their utilization into other valuable products. With this in view, it is interesting to explore the essential oil, and hexane extract of *Citrus limon* leaves for valuable bioactive compound profiling. Therefore, the current research is designed to explore the chemical composition, antioxidant potential, cytotoxicity, and *in silico* studies of essential oil and hexane extract from leaves of *Citrus limon* that will help to establish the scientific basis for its consumption as nutra/pharmaceutical.

Results and Discussion

Percentage Yield of Citrus limon Leaves Essential Oil (CLEO) and Hexane Extract

The present study showed that the average percentage yield of CLEO and hexane extract obtained by hydrodistillation was 0.59 and 0.50%, respectively. In medicinal plants, the contents and composition of essential oil are influenced by various conditions such as soil and climatic conditions, techniques used to grow, and way of harvesting and irrigation.[16]

Identification of Compounds in Citrus limon Leaves Essential Oil (CLEO) and Hexane Extract Using GC/MS

Twenty-five compounds were recognized in CLEO by GC/MS, as presented in *Table 1*. Major chemical compounds were limonene (60.52%), methyl palmitate (5.29%), 8,11-octadecadienoic acid methyl ester (2.94%),, elaidic acid methyl ester (3.72%), 3β-acetoxyolean-12-ene (1.69%), 2,6,10,14,18,22-tetracosahexaene (4.36%), methyl arachisnte (3.96%),, taraxasterol (2.35%) and *n*-eicosane (1.06%) (*Table 1*). Kabara[17] reported that antimicrobial activity against *Clostridium perfringens* and *Staphylococcus pyogenes* was shown by fatty acids such as oleic, stearic, palmitic, linoleic, myristic, and linolenic acids and affected the aflatoxin contamination directly or indirectly.[18] The earlier studies show that chemical compounds 2,6,10,14,18,22-tetracosahexaene identified in the *Wedelia* species essential oils have been used as an antioxidant, antibacterial and antifungal agents.[19] Methyl anarchiste present in the essential oil of *Callistemon comboynensis* leaves has been employed as an antimicrobial agent in food products.[20] . The literature also shows that, limonene found in citrus fruits such as lemon shows antimicrobial properties.[21]

On the other hand, in the hexane extract, *cis*-oleic acid (12.23%), hexadecanoic acid (2.65%), *cis*-9-octa-

Table 1. Components found in *Citrus limon* leaves essential oil (CLEO) by GC/MS analysis.

Retention time (min)	Retention indices (RI) (calculated)	Retention indices (RI) (literature)	Name of compounds	% area
1.93	331	333	*n*-Eicosane	1.06
9.99	567	569	Viminalol	2.30
11.64	1028	1030	Limonene	60.52
11.69	1211	1214	Moretenol	3.10
11.89	1483	1480	Taraxasterol	2.35
13.38	1891	1900	Nonadecane	t*
13.64	1902	1906	Methyl hexacosanoate	t
13.75	1925	1928	Methyl palmitate	5.29
13.97	2069	2072	8,11-Octadecadienoic acid, methyl ester	2.94
14.03	2085	2088	Methyl stearate	0.94
14.22	2108	2110	Elaidic acid, methyl ester	3.72
15.27	2173	2174	3-Methylheneicosane	t
16.54	2226	2231	Methyl arachisate	3.96
16.83	2279	2284	Aristolone	0.81
17.36	2299	2302	Methyl eicosanoate	0.60
17.46	2511	2530	Docosanoic acid, methyl ester	0.79
17.78	2733	2700	Heptacosane	t
18.35	2715	2729	Methyl lignocerate	0.81
18.73	2787	2790	2,6,10,14,18,22-Tetracosahexaene	4.36
20.84	2897	2900	Nonacosane	0.83
21.49	3327	3339	3β-Acetoxyolean-12-ene	1.69
21.62	3523	3525	Lupeyl acetate	t
24.34			N.I.*	t
25.11			N.I.	0.90
	Total	99.96		
	Total Monoterpene Content	60.52		
	Fatty Acid Methyl Ester	13.73		
	Terpenoids	11.5		
	Triterpene	2.30		
	Sesquiterpenoids	0.81		
	Alkane Hydrocarbons	1.89		
	Saturated Fatty Acids	0.81		
	Unsaturated Fatty Acids	3.72		
	Miscellaneous Compounds	0.79		
	Others	3.89		

*N.I. = Not identified (Compounds); t = trace; Where*t = trace; The retention indices were calculated relative to a homologous series of *n*-alkanes (C_8-C_{28}) injected under the same conditions.

decenoic acid (1.67%), 2H-1-benzopyran-2-one (1.18%), and phthalic acid (1.81%) were identified as major compounds (*Table 2*). Previous studies have shown that the long-chain fatty acids, i.e., oleic and linoleic acids, are the inhibitors of enoyl-acyl carrier protein reductase.[22]

From earlier reports, it has been found that the antibacterial activity is due to the presence of hexanoic acid in the hexane extract of the plant, which may be used for food preservation.[23] The hexane extract of neem leaves shows antifungal activity due to 2H-1-benzopyran-2-one, *cis*-9-octadecenoic acid, and phthalic acid.[24] GC/MS analysis of extract (oil) and bio-assays of crude extract of *Iris kashmiriana* showed that the 8,11-Octadecadienoic acid, methyl ester has the effects of lipid peroxidation in the serum in lactating women.[25] It was found that different secondary metabolites and bioactive phytoconstituents analysed by GC/MS are involved in anti-inflammatory, antimicrobial, antioxidant, and antiproliferative activities.[26]

Table 2. Components found in hexane extract of *Citrus limon* leaves by GC/MS analysis.

Retention time (min)	Retention indices (RI) (calculated)	Retention indices (RI) (literature)	Chemical compound	% area
1.93	327	333	Eicosane	t
6.83	1026	1030	Limonene	73.62
8.68	1481	1488	Cyclopentadecane	0.51
8.95	1881	1883	1-Hexadecanol	t
10.37	1837	1840	2H-1-Benzopyran-2-one	1.18
10.67	1861	1865	n-Octadecane	t
11.68	1915	1917	Phthalic acid	1.81
11.85	1931	1933	Tetradecanal	0.33
12.13	1962	1961	Hexadecanoic acid	2.65
12.28	2019	2022	Amyrolin	0.63
12.52	2081	2085	*cis*-9-Octadecenoic acid	1.67
12.97	2103	2100	Heneicosane	t
15.5	2106	2109	9-Octadecenoic acid	t
15.66	2130	2134	(1*E*,13*E*)-2,13-Octadecadien-1-ol	0.82
17.36	2171	2175	*cis*-oleic acid	12.23
	Total			99.95
	Total Monoterpene Content			73.62
	Long Chain Fatty Acids			16.88
	Secondary Metabolites			1.18
	Alkane Hydrocarbons			0.51
	Alcohols			0.82
	Aldehydes			0.33
	Other Organic Compounds			2.44
	Others			4.17

Where*t = trace; The retention indices were calculated relative to a homologous series of *n*-alkanes (C_8-C_{28}) injected under the same conditions.

Antioxidant Potential of CLEO and Hexane Extract

DPPH Radical Scavenging Activity and Percent Inhibition in Linoleic Acid Peroxidation

The antioxidant potential of CLEO and hexane extract in terms of free radical scavenging activity and percent inhibition in the linoleic acid peroxidation is presented in *Table 3*. The free radical scavenging activity of hexane extract and CLEO was analyzed by DPPH assay in a concentration-dependent manner. According to the results, CLEO has greater radical scavenging activity (69.64 %) than the hexane extract (67.55 %).

Table 3. Antioxidant activity of CLEO essential oils and hexane in terms of DPPH free radical scavenging activity and inhibition of peroxidation in a linoleic acid system.

Samples	DPPH free radical scavenging activity	Percentage inhibition (%)
Essential oil (CLEO)	69.64 ± 0.13	81.93 ± 0.19
Hexane	67.55 ± 0.10	50.34 ± 0.39
BHT	86.93 ± 0.19	89.53 ± 0.38

From the results, it has been concluded that the essential oils showed more antioxidant activity than hexane extract, whereas the percentage scavenging exhibited by synthetic antioxidant butylated hydroxy toluene (BHT) was 86.93 %.

Linoleic acid, upon oxidation, gives peroxides that oxidize Fe^{+2} to Fe^{+3}. Inhibition of linoleic acid oxidation is an important issue in food industries as it may deteriorate the products that smell badly at low threshold values.[27,28] The antioxidant activity of any sample is highly related to the inhibition of linoleic acid oxidation. The antioxidant action of essential oils and hexane extract of *Citrus limon* leaves assessed in terms of percent inhibition in the linoleic acid system is given in *Table 3*. The hexane extract showed a lower percent inhibition in linoleic oxidation (50.0 %) compared to the essential oils (81.0 %). The BHT used as control showed inhibition, i.e., 89.53 %. Previous studies documented that the oxidation of lipids affects the nutritional value of foods. The main byproducts formed from oxidation are unstable hydroperoxides which give rise to various secondary products such as alkanes, alcohols, aldehydes, and acids.[29] Phytocon-

stituents can donate a hydrogen atom to free radicals and inhibit the chain propagation reactions that occur during the oxidation of lipids.[30]

β-Carotene-linoleic Acid Assay

Bleaching of β-carotene with the linoleic acid system as antioxidant activity of *Citrus limon* leaves essential oil (CLEO), and hexane extract was analysed as shown in *Figure 1*. CLEO presented better antioxidant activity than the hexane extract of *Citrus limon* leaves. Supported by the results, the order of antioxidant activity was as follows: BHT > essential oils > hexane extract > control. β-carotene usually undergoes rapid discoloration due to the formation of free radicals in the absence of an antioxidant.[31] No earlier data exists in the literature regarding the antioxidant action of *Citrus limon* leaves essential oil (CLEO) and hexane extract using bleaching of the β-carotene-linoleic acid assay.

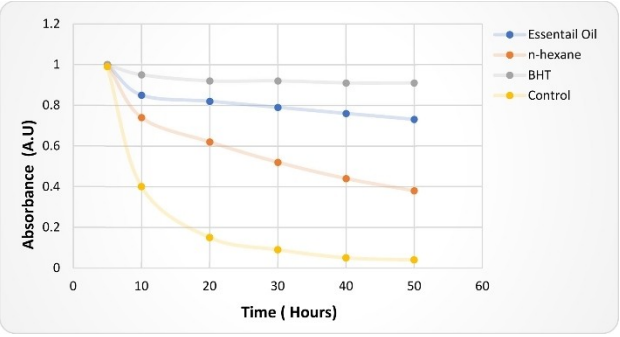

Figure 1. *β*-Carotene linoleic acid assay of *Citrus limon* leaves essential oil (CLEO) and hexane extract.

Antimicrobial Activity

In vitro Antimicrobial Studies by Zone of Inhibition and Minimum Inhibitory Concentration (MIC)

The results of disc diffusion and minimum inhibitory concentration (MIC) presented in this study revealed that CLEO and hexane extract from lemon offered good potential against chosen bacterial and fungal strains (*Table 4*). CLEO showed the highest inhibition zone (29.50 mm) and lowest MIC value (2.05 mg/ml) against *E. coli*, whereas the least inhibition zone (27.50 mm) and MIC value (3.50 mg/ml) by CLEO was noted for *B. subtilis*. No activity was shown by hexane extract and CLEO against *S. aureus* and *P. multocida*, respectively.

CLEO offered a better inhibition zone (26.90 mm) and the lowest MIC value (2.50 mg/mL) against *R. solani*. The hexane extract showed less activity against all the bacterial and fungal strains than CLEO. The reason behind this may be the presence of some potent bioactive in CLEO compared to hexane extract with better efficacy to inhibit the zone formation and less concentration. Several other compounds identified from essential oils (methyl palmitate, hexane, elaidic acid, methyl ester, nonacosane, methyl arachisate, taraxasterol, moretenol) and hexane extract (phthalic acid, hexadecanoic acid, oelsaurere, amyrolin, heneicosane, and cyclopentadecane) are also reported to show better antimicrobial activity against selected fungal and bacterial strains. Essential oils of plants show significant antimicrobial activity, and this is due to the synergistic properties of their components.[12]

In silico Studies

In silico studies show that moretenol, taraxasterol, oelsauere, elaidic acid, and vinimalol, although present

Table 4. Antimicrobial activity of *Citrus limon* leaves essential oils (CLEO) and hexane extract in terms of inhibition zone (mm) and MIC (mg/mL).

Samples inhibition zone (mm)	*Fungal strains*				*Bacterial strains*			
	A. flavus	*A. niger*	*R. solani*	*A. alternata*	*B. subtilis*	*P. multocida*	*S. aureus*	*E. coli*
CLEO	21.30 ± 0.17	N.D.*	26.90 ± 0.12	23.50 ± 0.12	27.50 ± 0.27	N.D.*	28.40 ± 0.22	29.50 ± 0.26
Hexane	17.50 ± 0.16	N.D.*	22.60 ± 0.17	19.51 ± 0.19	22.50 ± 0.19	15.50 ± 0.33	N.D.*	25.40 ± 0.21
†Standard	22.40 ± 0.15	28.10 ± 0.17	29.70 ± 0.12	24.90 ± 0.12	29.50 ± 0.26	27.30 ± 0.18	29.80 ± 0.24	28.20 ± 0.24
Samples	Fungal Strains				Bacterial Strains			
MIC (mg/mL)	*A. flavus*	*A. niger*	*R. solani*	*A. alternata*	*B. subtilis*	*P. multocida*	*S. aureus*	*E. coli*
CLEO	4.00 ± 0.11	N.D.*	2.50 ± 0.10	3.00 ± 0.11	3.50 ± 0.11	N.D.*	2.50 ± 0.10	2.05 ± 0.09
Hexane	4.50 ± 0.12	N.D.*	3.50 ± 0.11	4.00 ± 0.11	4.50 ± 0.12	5.00 ± 0.03	N.D.*	2.30 ± 0.10
†Standard	0.10 ± 0.02	0.05 ± 0.04	0.02 ± 0.01	0.06 ± 0.04	0.06 ± 0.04	0.13 ± 0.05	0.03 ± 0.02	0.08 ± 0.07

*N.D. = Not detected. † ciprofloxacin (antibacterial) and fungone (antifungal).

in small amounts (*Table 1* and *2*), may be enough to confer the antimicrobial potential to CLEO against *E. coli* and *S. aureus* (*Figures 2*–5, *Tables 5*–8). In the case of dihydroorotase from *E. coli*, moretenol was the best ligand with a binding energy of −8.17 kcal/mol and a dissociation constant of 1.03 μM (*Table 5*). The ligand binding site for moretenol consisted of His18, Arg20, Asn44, Tyr79, Tyr104, Pro105, Ala106, Asn107, Thr110, His114, Gly115, Val116, His139, Thr143, Cys221, Leu222, Asp250, Ala252, His254, Ala266, Gly267, and Cys268. Moretenol also interacts with zinc metal ions present in the enzyme's active site (*Table 5*). Taraxasterol and vinimalol also share a similar enzyme binding site, while Oelsauere binds to a separate approximate binding site consisting of Phe205, Val215, Arg216, Pro217, His218, Leu219, Pro253, His254, Ala255, Arg256, Lys259, Gly267, Cys268, Phe269, Leu332, Val333, Pro334, Phe335, Leu336, Ala337, and Glu339 (*Figure 2*). Vinimalol, taraxasterol, moretenol, and oelsauere share the same binding site when

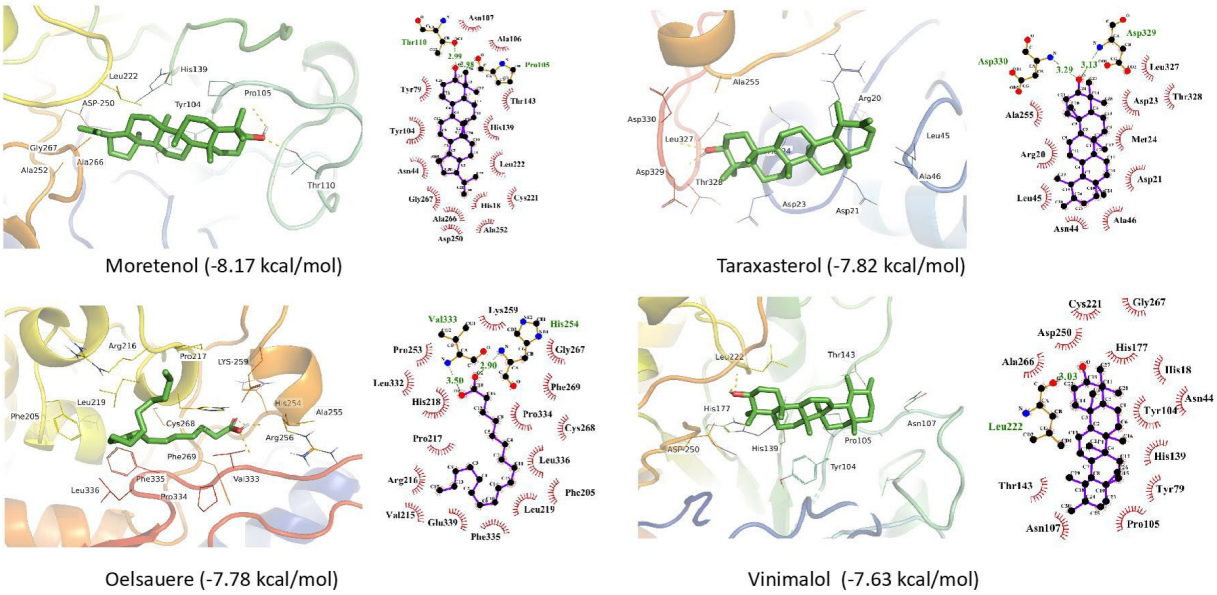

Figure 2. Moretenol, taraxasterol, oelsauere, and vinimalol docking against the Dihydroorotase from *E. coli*.

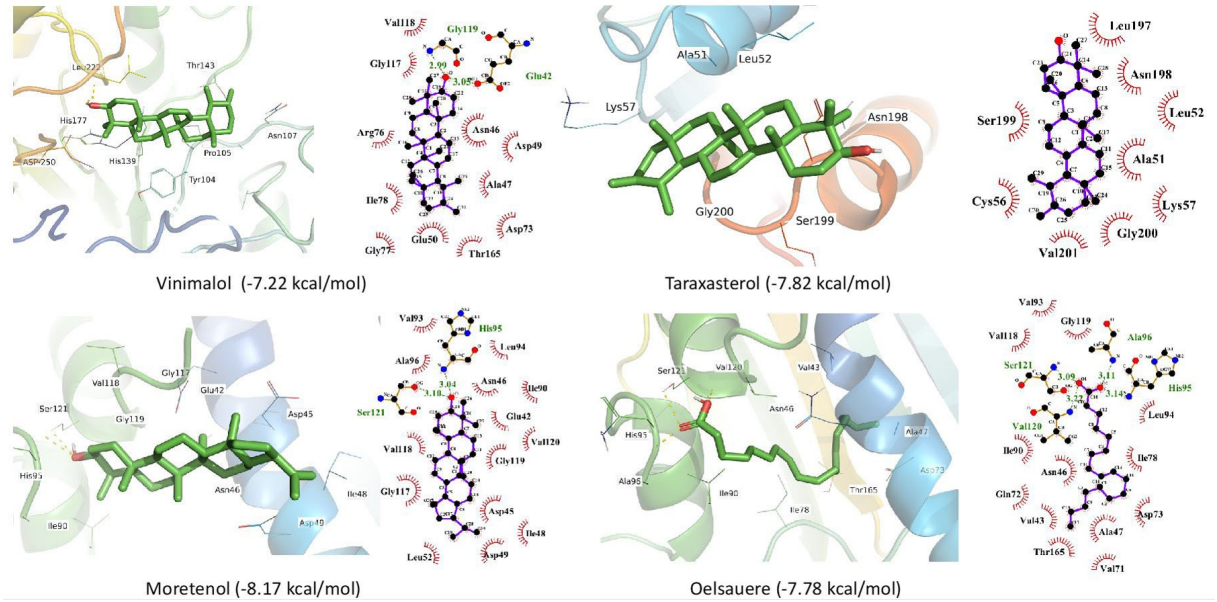

Figure 3. Vinimalol, taraxasterol, moretenol, and oelsauere docking against the DNA gyrase from *E. coli*.

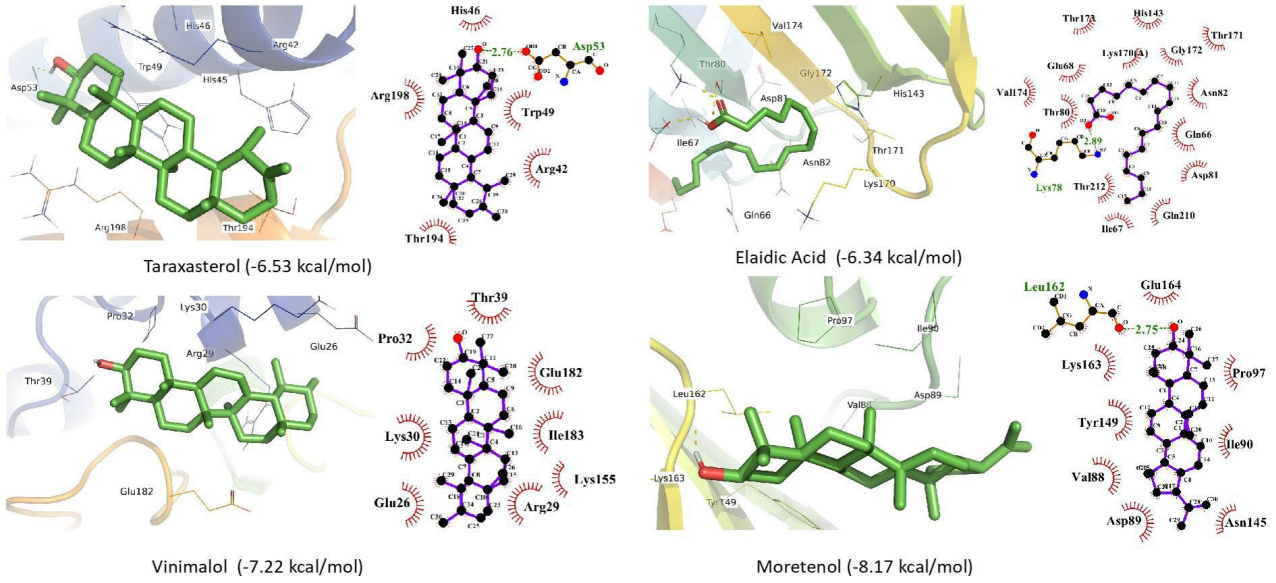

Figure 4. Taraxasterol, elaidic acid, vinimalol, and moretenol docking against the tyrosyl-tRNA synthetase from *S. aureus*

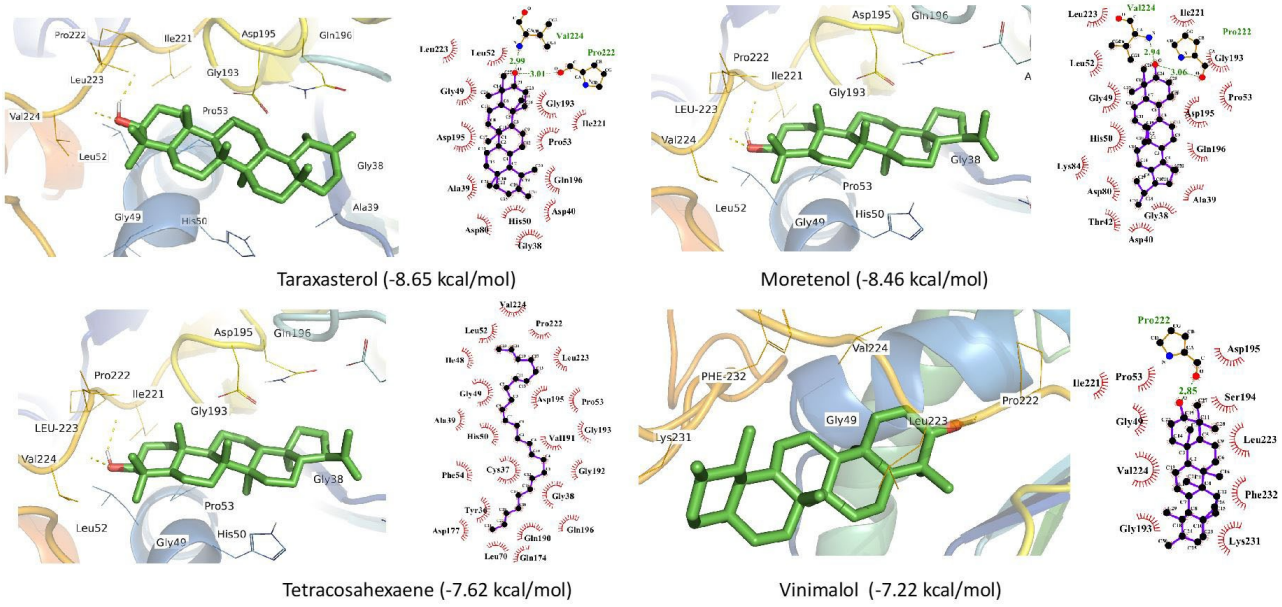

Figure 5. Taraxasterol, moretenol, tetracosahexaene, and vinimalol, and docking against the DNA gyrase from *S. aureus.*

binding to gyrase from *E. coli* (*Figure 3*). This binding site consists of amino acid residues including Val43, Asn46, Ala47, Glu50, Ile59, Val71, Gln72, Asp73, Ile78, Ile90, Met91, Val93, Leu94, His95, Ala96, Val118, Gly119, Val120, Ser121, Val122, Thr165, Met166, and Val167. Vinimalol shows the best binding energy of −7.22 kcal/mol with a dissociation constant of 5.10 μM (*Table 6*).

The three best ligands against the tyrosyl-tRNA synthetase and gyrase from *S. aureus* include taraxasterol, vinimalol, and moretenol (*Tables 7* and *8*). Taraxasterol shows binding energy of −6.53 and −8.65 with a dissociation constant of 16.4 and 0.45 μM (*Tables 7* and *8*) against tyrosyl-tRNA synthetase and gyrase, respectively. The binding site for tyrosyl-tRNA synthetase consisted of Glu41, Arg42, His45, His46, Trp49, Asp53, Thr194, Arg198, and Leu202 and for gyrase it was Cys37, Gly38, Ala39, Asp40, His47, Ile48, Gly49, His50, Leu52, Pro53, Asp80, Lys84, Tyr170, Gln174, Gly192, Gly193, Ser194, Asp195, Gln196,

Table 5. Selective phytochemicals docked against Dihydroorotase from *E. coli.*

Ligands	CID	Binding Energy (kcal/mol)	Dissociation Constant (μM)	Active site Residues
Moretenol	12309610	−8.17	1.03	HIS 18, ARG 20, ASN 44, TYR 79, TYR 104, PRO 105, ALA 106, ASN 107, THR 110, HIS 114, GLY 115, VAL 116, HIS 139, THR 143, CYS 221, LEU 222, ASP 250, ALA 252, HIS 254, ALA 266, GLY 267, CYS 268, ZN, ZN
Taraxasterol	115250	−7.82	1.85	ARG 20, ASP 21, GLY 22, ASP 23, MET 24, LEU 25, ASN 44, LEU 45, ALA 46, HIS 254, ALA 255, HIS 257, LEU 327, THR 328, ASP 329, ASP 330
Oelsauere	445639	−7.78	1.97	PHE 205, VAL 215, ARG 216, PRO 217, HIS 218, LEU 219, PRO 253, HIS 254, ALA 255, ARG 256, LYS 259, GLY 267, CYS 268, PHE 269, LEU 332, VAL 333, PRO 334, PHE 335, LEU 336, ALA 337, GLU 339
Vinimalol	73170	−7.63	2.54	HIS 18, ASN 44, TYR 79, TYR 104, PRO 105, ASN 107, THR 110, HIS 139, THR 143, HIS 177, CYS 221, LEU 222, PRO 223, ASP 250, ALA 252, HIS 254, ALA 266, GLY 267, ZN 400, ZN 401

Table 6. Selective phytochemicals docked against DNA gyrase from *E. coli.*

Ligands	CID	Binding Energy (kcal/mol)	Dissociation Constant (μM)	Active site Residues
Vinimalol	73170	−7.22	5.10	GLU 42, ASP 45, ASN 46, ALA 47, ASP 49, GLU 50, ASP 73, GLY 75, ARG 76, GLY 77, ILE 78, PRO 79, ILE 90, HIS 95, GLY 117, VAL 118, GLY 119, VAL 120, SER 121, ARG 136, GLY 164 A THR 165
Taraxasterol	115250	−6.91	8.66	ALA 51, LEU 52, GLY 54, CYS 56, LYS 57, LEU 197, ASN 198, SER 199, GLY 200, VAL 201, SER 202
Moretenol	12309610	−6.61	14.20	GLU 42, ASP 45, ASN 46, ILE 48, ASP 49, LEU 52, ILE 90, VAL 93, LEU 94, HIS 95, ALA 96, HIS 116, GLY 117, VAL 118, GLY 119, VAL 120, SER 121
Oelsauere	445639	−6.49	17.4	VAL 43, ASN 46, ALA 47, GLU 50, ILE 59, VAL 71, GLN 72, ASP 73, ILE 78, ILE 90, MET 91, VAL 93, LEU 94, HIS 95, ALA 96, VAL 118, GLY 119, VAL 120, SER 121, VAL 122, THR 165, MET 166, VAL 167

Table 7. Selective phytochemicals docked against tyrosyl-tRNA synthetase from *S. aureus.*

Ligands	CID	Binding Energy	Dissociation Constant (μM)	Active site Residues
Taraxasterol	115250	−6.53	16.4	GLU 41, ARG 42, HIS 45, HIS 46, TRP 49, ASP 53, THR 194, ARG 198, LEU 202
Elaidic Acid	637517	−6.34	22.5	GLN 66, ILE 67, GLU 68, LYS 78, THR 80, ASP 81, ASN 82, HIS 143, LYS 170, THR 171, GLY 172, THR 173, VAL 174, GLN 210, ILE 211, THR 212, SER 226
Vinimalol	73170	−6.26	25.7	GLU 26, ARG 29, LYS 30, ARG 31, PRO 32, THR 39, LYS 155, GLU 182, ILE 183, THR 185
Moretenol	12309610	−6.21	27.9	VAL 88, ASP 89, ILE 90, PRO 97, ASN 145, TYR 149, ASP 161, LEU 162, LYS 163, GLU 164

Asn199, Ile221, Pro222, Leu223, Val224, and Phe232 (*Tables 7* and *8*). Taraxasterol show very tight binding with *S. aureus* gyrase which is in agreement with *in vitro* studies where CLEO shows a zone of inhibition of 28.40 mm. The docking analysis of *N*-(benzo[*d*]thiazol-2-ylcarbamothioyl)-2/4-substituted benzamides against the protein *E. coli's* dihydroorotase showed a binding energy of −7.4 kcal/mol, while benzothiazole and substituted benzothiazole compounds showed highest binding affinity of −5.02 and −4.57 kcal/mol, respectively.

The active site included Leu222, Ala266, His254, Arg20, Asn44, and His139. The triazinane and oxadiazinanes showed significant antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli*, and *S. typhi*, whereas molecular docking analysis of these compounds

Table 8. Selective phytochemicals docked against tyrosyl-tRNA synthetase from *S. aureus*.

Ligands	CID	Binding Energy	Dissociation Constant (μM)	Active site Residues
Taraxasterol	115250	−8.65	0.45	CYS 37, GLY 38, ALA 39, ASP 40, HIS 47, ILE 48, GLY 49, HIS 50, LEU 52, PRO 53, ASP 80, LYS 84, TYR 170, GLN 174, GLY 192, GLY 193, SER 194, ASP 195, GLN 196, ASN 199, ILE 221, PRO 222, LEU 223, VAL 224, PHE 232
Moretenol	12309610	−8.46	0.62	GLY 38, ALA 39, ASP 40, THR 42, HIS 47, ILE 48, GLY 49, HIS 50, LEU 52, PRO 53, ASP 80, LYS 84, ARG 88, TYR 170, GLY 192, GLY 193, SER 194, ASP 195, GLN 196, ILE 221, PRO 222, LEU 223, VAL 224, PHE 232
Tetracosahexaene	57417215	−8.15	1.07	TYR 36, CYS 37, GLY 38, ALA 39, ASP 40, ILE 48, GLY 49, HIS 50, LEU 52, PRO 53, PHE 54, LEU 70, GLY 72, THR 75
Vinimalol	73170	−7.51	3.14	HIS 47, GLY 49, LEU 52, PRO 53, GLY 193, SER 194, ASP 195, ILE 221, PRO 222, LEU 223, VAL 224, THR 225, LYS 226, GLY 229, LYS 230, LYS 231, PHE 232

against DNA gyrase showed the highest binding energy of −9.37 kcal/mol.

Cytotoxicity Studies by Haemolytic Activity

The cytotoxic effect of CLEO and hexane extract was evaluated in terms of the percent lysis of red blood cells. It was observed that 99.64% lysis was shown by the positive control (Triton X-100), whereas no lysis of RBCs was observed by the phosphate buffer saline (PBS). Whereas the haemolytic activity of CLEO and hexane extract was 2.46 and 5.75%, respectively. The haemolytic activity less than 10% is related to lower cytotoxicity and safe.[5,32,33] The RBCs% lysis for CLEO is (2.46%), which is considered as safe, thus supports its use in cosmetics and nutra-pharmaceutical industries. The current study revealed that the antioxidant, and antimicrobial activity of the CLEO was greater than that of the hexane extract.

Conclusion

The current study's findings revealed that hexane extract and essential oil of *Citrus limon* leaves possess potent antioxidant activities in terms of DPPH scavenging power and linoleic oxidation. The percentage scavenging of both extracts was found to be in the range of 67.55 to 69.64%, while percent inhibition in linoleic acid oxidation was assessed to range from 50.34 to 81.93%. The antimicrobial activity of CLEO was found to be better against bacterial and fungal species than hexane extract. The plant samples showed a hemolytic activity of 2.46 to 5.75%, whereas less than 10% activity is considered relatively safe for RBCs. Based on these results, it may be concluded that *Citrus limon* leaves agro waste can be beneficial if modified into essential oil and extract forms and thus utilized in cosmetics, food industries, and pharmaceuticals. Being a good antioxidant and antimicrobial activity and safe cytotoxicity, *Citrus limon* leaves essential oils and extracts could be used in herbal medicine.

Experimental Section

Essential Oil and Hexane Extracts of Lemon Leaves

Fresh leaves of lemon were collected from the urban area of Mandi Bahaudin, Pakistan, in August 2018. The plant was recognized and authenticated by Dr. Muhammad Haneef (Taxonomist), Botany Department, University of Sargodha. A voucher specimen (CHEM-07/18) was submitted to the University Herbarium.

The leaves were washed, air-dried, and ground into fine powdered form. Isolation of essential oil was executed using the Clevenger apparatus. The collected *Citrus lemon* essential oil (CLEO) was stored at 4 °C until further analyses. Whereas hexane extract was obtained by the Soxhlet apparatus using the method of Mahesh and Satish[34] with some modifications.

GC/MS Analysis of CLEO and Hexane Extract

The apparatus used for the GC/MS analysis consisted of a capillary column (30 m × 0.25 mm × 0.25 μm) with an electron ray of 70 eV. Helium gas (1 mL/min) was used as a transporter, and the temperature was maintained at 200 °C. There was a rise in temperature at the speed of 3 °C/min up to 150 °C, and after 3 min, the temperature was increased up to 280 °C and kept

constant. The total running time for the GC/MS was 30 min. To compute the comparative percentage of each component, the assessment was done between its average climax regions to the total area.[35] The retention indices were calculated relative to a homologous series of *n*-alkanes (C_8-C_{28}) injected under the same conditions.[14]

Evaluation of the Antioxidant Activity of CLEO and Hexane Extract

DPPH free radical scavenging activity

The antioxidant action of the CLEO and hexane extract was estimated by measuring their ability to scavenge DPPH radicals. The DPPH assay was carried out as the method described by Mimica-Dukic et al.[36]

Scavenging (%) of free radicals was calculated by using the following formula:

$$\text{Scavenging } (\%) = 100 \times (A_{blank} - A_{sample}/A_{blank})$$

Where A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the test essential oils/compounds.

Percent Inhibition in the Linoleic Acid System

The estimation of antioxidant activity of essential oils and hexane extract was also observed in terms of percent inhibition of peroxides in the linoleic acid system as described in an already reported method by Iqbal et al. with minor modification.[37] BHT was used as a positive control in this experiment.

Percent inhibition of linoleic acid oxidation was found from this formula:

$$\% \text{ Inhibition of peroxide} = 100\text{-}[(\text{Abs. increase of sample}/\text{Abs. increase of control} \times 100]$$

β-Carotene-Linoleic Acid Assay

β-carotene-linoleic acid assay of both CLEO and hexane extract was also evaluated by calculating the inhibition of the conjugated diene hydroperoxides from the linoleic acid oxidation as described by Kulisic, et al.[38] The absorbance of the solution was recorded at 490 nm, and the BHT was used as standard.

Cytotoxicity Studies

Cytotoxicity was assessed by the haemolytic activity of CLEO and hexane extract samples by the method described by Riaz et al. and Powell et al.[27,28] For every analysis, the positive control was 0.1% Triton X-100, and the negative control was phosphate buffer saline (PBS) solution.

Evaluation of Antimicrobial Studies

In Vitro Antimicrobial Studies

An *in vitro* study was performed to estimate the antimicrobial action of hexane extract and CLEO, at a concentration of 10 mg/mL with selected bacterial and fungal strains by the disc diffusion method.[39] Minimum inhibitory concentrations (MIC) were observed modifying previously described methods.[40,41] Ciprofloxacin and fungone (1 mg/mL) were standard drugs.

In silico Studies

For the *in silico* determination of the antimicrobial potential of the hexane extract and CLEO, a structural database of the major isolated compounds (from GC/MS analysis) was prepared. The ligands structures were either downloaded from ChemSpider or PubChem databases and optimized using molecular mechanic (MM) force field in Avogadro[42] and YASARA software 20.7.4.[43] The crystal structure of Dihydroorotase, DNA gyrase enzyme from *Escherichia coli*, tyrosyl-tRNA synthetase, and DNA gyrase from *Staphylococcus aureus* were obtained from Protein Data Bank (PDB) as PDB IDs 2EG7, 1KZN, 1JIJ and 3G7B, respectively. The ligand database was screened using Autodock-LGA in the virtual screening module of YASARA software using the parameters described earlier.[44–46] LigPlus[47] and PyMol were used to obtain ligand-protein interactions.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

Muhammad Riaz, Rahman Qadir and Muhammad Tahir Akhtar performed the experiments. Muhammad Misbah ur Rehman, Farooq Anwar, and Rida Eman analyzed the data and wrote the article. Muhammad Fayyaz ur Rehman contributed to the samples/reagents/materials/analysis tools and analyzed the data. Muhammad Safwan Akram edited the manuscript and aided the design of experiments.

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