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Journey of Limonene as an Antimicrobial Agent

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Abstract

Injudicious consumption of antibiotics in the past few decades has arisen the problem of resistance in pathogenic organisms against most antibiotics and antimicrobial agents. Scenarios of treatment failure are becoming more common in hospitals. This situation demands the frequent need for new antimicrobial compounds which may have other mechanisms of action from those which are in current use. Limonene can be utilized as one of the solutions to the problem of antimicrobial resistance. Limonene is a naturally occurring monoterpene with a lemon-like odor, which mainly present in the peels of citrus plants like lemon, orange, grapefruit, etc. The study aimed to enlighten the antimicrobial properties of limonene as per previous literature. Advantageous contributions have been made by various research groups in the study of the antimicrobial properties of limonene. Previous studies have shown that limonene not only inhibits disease-causing pathogenic microbes, however, it also protects various food products from potential contaminants. This review article contains information about the effectiveness of limonene as an antimicrobial agent. Apart from antimicrobial property, some other uses of limonene are also discussed such as its role as fragrance and flavor additive, as in the formation of nonalcoholic beverages, as solvent and cleaner in the petroleum industry, and as a pesticide. Antibacterial, antifungal, antiviral, and anti-biofilm properties of limonene may help it to be used in the future as a potential antimicrobial agent with minimal adverse effects. Some of the recent studies also showed the action of limonene against COVID-19 (Corona virus). However, additional studies are requisite to scrutinize the possible mechanism of antimicrobial action of limonene.

Keywords: Antibacterial, anti-biofilm, antifungal, antiviral, bioactive compound, limonene, COVID-19

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INTRODUCTION

Antibiotic resistance is emerging very rapidly because of the inappropriate or misuse of antimicrobials which is facilitated because of their accessibility over the counter, without prescription, and through unregulated delivery chains[1]. Antimicrobial agents also comprise adverse side effects along with microbial resistance. Hence, the interest of researchers is turned towards ethnopharmacology. Bioactive compounds are natural and ubiquitous in most plants and easily accessible to humans. Plant extracts provide immeasurable occasions for novel drug findings due to the exuberance of multifarious chemicals. Hence, thousands of obtainable medicinal phytochemicals are safe and more effective alternatives, for this reason, coupled with advancing microbial resistance to synthetic drugs; ethnopharmacology is swiftly gaining world acknowledgment[2,3]. In countries like Japan forest bathing trips are very popular and consider natural chemotherapy. Forest bathing trips include a trip to a forest for recreation and relaxation while breathing in volatile substances which are antimicrobial compounds derived from trees[4]. One of such components is limonene, which is one of the main constituents present in aromatic plants and the most common terpene present in nature. It is mainly found to be present in essential oils of peels of *citrus* spp. like lemon, orange, grapefruit, lime, and mandarin, etc. D-limonene is generally recognized as safe (GRAS) to be utilized as a flavoring agent. Due to its citrus fragrance, it is extensively used as a flavor and fragrance additive in soaps, perfumes, and chewing gums[5]. Limonene is also found to be present in many fruits, vegetables, meats, spices, and other food items[6]. Limonene is also popular for its various properties like antibacterial, antifungal, antiviral, and anti-biofilm. In the present study, the above-mentioned properties of limonene discussed and proved experimentally in various studies are included and reviewed.

What is Limonene?

Limonene is also known by some other synonyms like D-(+)-limonene, (+)-limonene, (R)-limonene, (R)-(+)-limonene, cajeputene, carvene, cinene, (+)-dipentene, etc. The molecular formula of limonene is $C_{10}H_{16}$ and molecular weight is 136.24. At room temperature limonene is a colorless liquid having lemon-like odor with a melting point temperature of 74.3°C, boiling point temperature of 175.5-176°C, and density of 0.8411g/ml (at 20°C) and 0.8402g/ml (at 25°C). It is slightly soluble in water (13.8mg/L at 25°C) and easily soluble in acetone, dimethyl sulfoxide, ethanol, benzene, carbon tetrachloride, diethyl ether, and petroleum ether. Limonene also gets oxidized when comes in contact with air and forms various oxidation products like carvone, limonene oxide, carveol, and limonene hydroperoxides hence it should be stored away from light and air. It is found to be present in peels of various citrus fruit species like orange, grape, lemon, and can be produced commercially from alkali treatment and steam distillation of citrus peel and pulp which remain after juice and cold-pressed oil production[7-10].

Uses of Limonene

Limonene is used as a flavor and fragrance additive in perfumes (0.005% and 1%), soaps, beverages, food, and household cleaning products for nearly 50 years. It is also used in the formation of nonalcoholic beverages (31ppm), ice creams (68ppm), candy (49ppm), baked goods (120ppm), sweets, gelatin, puddings (48-400ppm), and chewing gums (2300ppm). In the petroleum industry, it has been used as solvent and cleaner, in the transdermal application of medicines it is used as an additive to increase the penetration of active substance, it is also used as a degreasing agent before the lacquering of industrial products (30%), in the electronic industry for cleaning of printed circuits (50-100%) and for cleaning print cylinders in printing work. Pesticide, insect repellent, and dog/cat repellent are also well-known uses of limonene[6,7,9,10].

Antimicrobial activities of Limonene

Antiviral

Many viral diseases e.g. caused by Herpes simplex virus (HSV), influenza virus, and HIV are known as life-threatening. However various antiviral drugs against HIV, HSV, influenza, and other pathogenic viruses have been developed but the antecedence of these antiviral drugs is the potential and side effects of these drugs. There is still an urgent need for the development of new anti-viral agents which can fulfill all the drawbacks possessed by different antiviral drugs. Limonene can be used as a suitable anti-viral agent as some of the previous studies have emphasized its anti-viral properties.

Anti-viral properties of limonene were studied against human pathogenic (Herpes simplex and influenza virus) as well as plant pathogenic viruses (tobacco mosaic virus). The anti-viral property of limonene against the Herpes simplex type 1 virus was found as complete inhibition of HSV1 at 25µg/ml concentration of limonene was observed. Limonene inactivated the virus in the early phase of virus multiplication[11]. Cytotoxic concentration value (CC_{50}) of limonene at 1155µg/ml against HSV1 was also observed in another study[12]. Inhibition of the herpes simplex virus was observed by the formation of plaque on the kidney Vero cells[13]. On the other hand, the anti-viral property of *Citrus deliciosa*[14] and *Citrus reshni*[15] essential oils which contain limonene as the major component was also observed against the H5N1 virus. Anti-viral property of limonene against plant pathogenic virus, Tobacco mosaic virus (TMV) was also observed and it was found that TMV was inhibited by more than 45% after limonene treatment[16].

Apart from influenza and other viruses limonene also identified as the inhibitor of the SARS corona virus as described in many studies. The SARS corona virus has protein S a specific binding site for the angiotensin-converting enzyme 2 (ACE2) which serves as an entry point into the host cell. Limonene can act as the inhibitor for ACE2 target which is verified by performing docking studies[17]. Mechanism of action of herbal essential oils against viruses is either they coat the glycol proteins (potent of viral sites) of viruses or they bind with the human cell receptors (respiratory cells ACE- 2) which lead to the nonspecific and nonproductive binding of the virus particle to host cells which hinder the virus from infection. Some of the herbal essential oils which have antiviral or virucidal properties such as lavender oil, peppermint oil, and eucalyptus oil have limonene as the component[18]. ACE-2 inhibition property of limonene as a receptor for SARS-CoV-2 can prevent the invasion of SARS-CoV-2/COVID-19 into the human body[19,20]. Immunomodulatory, anti-inflammatory, and anti-viral properties of limonene can help limonene to limit the severity and succession of the COVID-19. Hence, limonene can act as a possible candidate against infection, immunity, and inflammation in COVID-19[21]. An oil blend was synthesized for the investigation of the effectiveness of the oil for the treatment in patients having COVID-19 like symptoms. The oil consisted of limonene as one of the chief components which provided anti-viral property to the oil. Limonene is known to restrain exclusively the viral DNA polymerase during the reproduction cycle when new viral DNA is synthesized. Hence, the oil was found as an effective remedial measure for COVID-19[22]. Limonene was also used for the quantitative evaluation of olfactory dysfunction in asymptomatic COVID-19 carriers[23].

Antibacterial

Antibacterial properties of limonene are well known and discussed in several reports against various bacterial species. Limonene is known to be active against many pathogenic bacteria involved in different diseases such as respiratory or skin diseases whereas some are known as contaminants causing contamination in the food industry. Synergistic activity of the combination of limonene enantiomers (D and R) and with other compounds has also been very well documented in many studies. Some of the studies have also highlighted the evaluation of chemical compositions of various essential oils from different plant materials and observed limonene as a major constituent and the reason for antibacterial activity shown by that essential oil. On the other hand, several methods were also reported in various studies to design nano-emulsions of limonene to be utilized in drug delivery, etc. Antibacterial actions of limonene against various bacterial species in various studies are summed up in Table 1.

Apart from the study on the antibacterial action of limonene, the mechanism of antibacterial action of limonene was also explained in some of the studies. A β-barrel protein (LptD) is an essential protein of the outer membrane present in lipopolysaccharide (LPS) assembly, depletion of this protein leads to the increase in membrane permeability of the bacterial cell. Attenuated total reflectance infrared microspectroscopy results after the treatment of *E. coli* cells with limonene showed the damage of LPS and altered outer membrane permeability was also observed in the study. It was concluded that the damage of LPS is the mechanism of inactivation by

Table 1. Antibacterial activity of Limonene

S.No.	Study	Concentration	Effective against	References
	Antibacterial activity of Limonene used in pure form			
1	Action of oxidized d-limonene on microorganisms	Pure	*S. elipsoideus, W. hansenula* and *O. lactis*	[33]
2	Antibacterial activity of limonene against food borne bacteria	98%	*E. coli, S. aureus, S. senftenberg* and *Pseudomonas* spp.	[34]
3	Antibacterial action of limonene from Douglas Fir against *Bacillus thuringiensis*.	Pure	*Bacillus thuringiensis*	[35]
4	Antibacterial action of limonene against 16 bacterial isolates	Pure	Nine gram positive and 16 gram negative bacteria	[36]
5	Antibacterial action of limonene against respiratory tract pathogens	Pure	*S. pyogenes, S. aureus, S. pneumoniae, H. influenzae* and *E. coli*	[37]
6	Antibacterial action of two enantiomers of limonene	Pure	*E. coli, E. faecalis, S. typhi, S. aureus, M. smegmatis, S. mutans, B. subtilis* and *E. aerogenes*	[38]
7	Anti-listerial action of limonene as essential oil component of Conifers	Pure (98%)	*L. monocytogenes*	[39]
8	Antibacterial action of limonene as component of Juniper berry oil	Pure	*S. marcescens, E. cloace, K. pneumoniae, A. baumani and S. aureus*	[40]
9	Antimicrobial activity of limonene	Pure	*E. coli, S. typhi, S. enteritidis, M. luteus, S. aureus, S. epidermidis, P. tolaasii*	[41]
10	Antibacterial action of limonene against *E. coli*	Pure	*E. coli*	[42]
11	Antibacterial action of limonene against fruit juice spoiling bacteria	Pure	*Lactobacillus plantarum, Lactobacillus brevis*, and *Bacillus coagulans*	[43]
12	Antibacterial action of limonene from commonly consumed medicinal herbs against human pathogenic bacteria	Pure	*M. flavus, B. subtilis, S. epidemidis, S. aureus, S. enteritidis, S. typhimurium, E. coli, E. cloacae* and *L. monocytogenes*	[44]
13	Antibacterial action of limonene isolated from Umbelliferae and Labiatae plants	Pure	*P. aeruginosa, P. mirabilis, K.pneumoniae, A. baumannii, S. aureus, E. faecalis and B. subtilis*	[45]
14	Antimicrobial property of limonene	Pure	Unknown organisms	[46]
15	Antibacterial action of limonene from *Wedelia prostrata* plant	Pure	*P. aeruginosa, E. coli, B. subtilis, S. aureus*	[47]
16	Antibacterial action of limonene against food related microorganisms	Pure	*B. cereus, E. coli, P. anomala* and *P. putida*	[48]

Table 1. Cont...

S.No.	Study	Concentration	Effective against	References
	Antibacterial activity of Limonene used in pure form			
17	Antibacterial action of limonene from indigenous plants against some clinical isolates	Pure (98%)	*S. aureus, S. pneumoniae, S. typhi, E. coli, P. aeruginosa, S. dysenteriae, and P. mirabilis*	[49]
18	Antibacterial action of limonene against *E. coli*	Pure (97%)	*E. coli*	[25]
19	Use of limonene as a biocidal agent	Pure	*S. aureus*	[50]
20	Antibacterial action of limonene from *Monarda punctata* essential oil against respiratory tract pathogen	Pure	*S. pyogenes, S. aureus, S. pneumoniae, H. influenzae* and *E. coli*	[51]
21	Antibacterial action of limonene against food-borne pathogens	Pure (95%)	*Y. enterocolitica, S. aureus* and *L. monocytogenes*	[52]
22	Antibacterial activity of limonene from *Artemisia capillaris* essential oil against respiratory tract infection-causing pathogens	Pure	*S. pyogenes, S. aureus, S. pneumoniae, K. pneumoniae, E. coli* and *H. influenzae*	[53]
23	Antibacterial activity of limonene against *Streptococcus uberis*	Pure	*S. uberis*	[54]
24	Inhibition of *P. aeruginosa* involved in biofilm formation by limonene from Citrus species	Pure	*P. aeruginosa*	[55]
25	Antibacterial action of Fish Gelatin-Chitosan edible films supplemented with D-Limonene	Pure (≥ 95%)	*E. coli*	[56]
26	Antibacterial action of limonene against fish pathogenic bacteria	Pure >99% concentrated	Seven gram negative and nine gram positive bacteria	[57]
	Synergistic activity of Limonene			
27	Antimicrobial action of limonene ennatiomers and 1,8-cineol alone and in combination	Pure (97%)	*S. aureus, B. cereus, E. faecalis, E. coli, P. aeruginosa, K. pneumoniae* and *M. catarrhalis*	[58]
28	Synergistic combination of limonene with high hydrostatic pressure for preservation of fruit juices	97% (Pure)	*E. coli* and *L. monocytogenes*	[59]
29	Synergistic antibacterial property of limonene in combination with heat treatment and its use in food preservation	97% (Pure)	*E. coli*	[24]
30	Antibacterial action of terpenes in combination with antibiotics against *M. tuberculosis*	Pure (98%)	*M. tuberculosis*	[60]

Table 1. Cont...

S.No.	Study	Concentration	Effective against	References
	Nanoemulsion Of Limonene			
31	Antibacterial action of limonene used in nanoemulsion	Pure	*E. coli* and *L. delbrueckii*	[61]
32	Effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion	Pure	*E. coli, S. aureus* and *B. subtilis*	[26]
33	Antibacterial action of nisin and D-limonene containing organogel-nanoemulsion	Pure	*E. coli, S. aureus* and *B. subtilis*	[27]
34	Combined anti-listerial action of nanoemulsion of d-limonene and nisin	Pure	*L. monocytogenes*	[62]
35	Improving antimicrobial action of D-limonene by using novel organogel-based nanoemulsion	Pure	*E. coli, S. aureus* and *B. subtilis*	[28]
36	Antibacterial Cyclodextrin/ Limonene-Inclusion Complex Nanofibrous Webs	Pure (97%)	*E. coli* and *S. aureus*	[63]
	Antibacterial activity of essential oils containing Limonene			
37	Antibacterial action of *C. carvi* essential oil containing limonene	18.2% (second highest)	14 gram negative and 4 gram positive bacteria	[64]
38	Antibacterial action of essential oils from Korean citrus species	55.4 to 91.7% (Major)	*P. acnes* and *S. epidemidis*	[65]
39	Antibacterial action of *Pimpinella puberula* (DC.) Boiss. aerial parts essential oil	21.7-82.4% (Major)	*Pseudomonas, Y. enterocolitica, B. cereus, M. luteus, B. subtilis* and *S. aureus*	[66]
40	Antibacterial activity of essential oil of *Lantana camara* aerial parts	16.9% (second highest)	*E. coli, P. aeruginosa, S. aureus, S. faecium, M. leutus* and *L. monocytogenes*	[67]
41	Antibacterial action of *Crassocephalum rubens* leaf extract	48.8% (Major)	*S. aureus, Streptococcus faecalis Escherichia coli* and *Salmonella typhi*	[68]
42	Activity of Citronella oil against spoilage bacteria of semi dried round scad	86% (Major)	*Pseudomonas spp., S. aureus* and *Klebsiella* spp.	[69]
43	Antibacterial action of limonene present in aerial parts of *Nepeta pungens* Benth essential oil	12% (second highest)	*S. aureus*	[70]
44	Antibacterial action of limonene as a component of essential oil of *Aloysia polystachya* leaves	20.22-14.65% (second highest)	*E. coli* and *S. aureus*	[71]

Table 1. Cont...

S.No.	Study	Concentration	Effective against	References
45	Antibacterial action of Makrut lime essential oil against respiratory tract pathogens	40.65% (Major)	*A. baumannii, Streptococci, H. influenzae, M. catarrhalis, S. aureus* and *S. pneumoniae*	[72]
46	Antibacterial action of sweet oranges peel essential oil	95.62% (Major)	*S. typhi, S. aureus, B. cereus, P. aeruginosa* and *Shigella* spp.	[73]
47	Antibacterial action of essential oil of Sweet lime	95.98% (Major)	*S. aureus, B. subtilis, B. cereus* and *L. acidophilus*	[74]
48	Antibacterial action of *Ammodaucus leucotrichus* Coss. and Dur. seeds essential oil	23.89%	*B. subtilis* and *E. coli*	[75]
49	Antibacterial action of leaves and fruit peel essential oil of *Citrus sinensis* Linn.	30.1% (second highest)	*S. aureus* and *E. coli*	[76]
50	Antibacterial action of essential oil of *Citrus paradisi* and *Citrus grandis*	96.06% and 55.74%	*P. aeruginosa, E. coli, S. enterica, S. aureus* and *S. faecalis*	[77]
51	Antibacterial activity of *Citrus paradise* essential oil	92.6%	*L. plantarum, L. mesenteroides, L. lactis, S. aureus* and *E. coli.*	[78]
52	Antibacterial action of essential oil components of orange peel	73.9-97% (Major)	*S. aureus, L. monocytogenes* and *P. aeruginosa*	[79]
53	Antibacterial action of essential oils from *Melissa officinalis* leaves	60.70% (Major)	*B. cereus, S. aureus, E. coli* and *S. enterica*	[80]
54	Antibacterial activity of *Pistacia terebinthus* essential oils	13.95-46.29% (second highest)	*S. aureus*	[81]
55	Antimicrobial action of black pepper, cardamom, cumin and coriander essential oil.	16.924%, 0.07%, 0.565% and 2.526%	*S. aureus, P. vulgaris, Salmonella* sp. and *E. coli*	[82]
56	Antibacterial action of *Citrus medica* L. var proper leaf essential oil	11.54% (third highest)	*S. aureus* and *P. acne*	[83]
57	Antibacterial action of essential oils from Citrus species	66-93%	*E. coli, B. cereus, S. aureus, S. typhimurium, L. monocytogenes* and *E. faecalis*	[84]
58	Antibacterial action of essential oil of lemon peel	46.93% (Major)	*S. aureus*	[85]
59	Antibacterial action of the essential oil of *Citrus aurantifolia* L. leaves	63.35% (Major)	*E. coli* and *S. aureus*	[86]
60	Antibacterial action of essential oils from *Lamiaceae* Family Plants	0.17-13.5%	*S. lutea, E. coli* and *S. saproph.*	[87]

(+)-limonene and cell envelopes are the important target of limonene[24]. It was also explained that the mechanism of inactivation of *E. coli* cells by the treatment of limonene depends on the physiological state of the cell and the concentration of limonene. Exponentially growing *E. coli* cells were inactivated by limonene at the concentration of 2,000µL/L and the mechanism of bacterial inactivation was observed to be the Fenton-mediated hydroxyl radical formation which leads to the oxidative DNA damage of the bacterial cell. On the other hand, 4,000µL/L concentration of limonene caused a change in the membrane permeability of the cells and it was observed that at higher concentrations, inactivation of the bacterial cell is related to the altered membrane permeability[25]. Treatment of limonene nano-emulsion also showed the complete collapse of the cell structure which leads to the cell lysis and release of intracellular material from inside of the cell. The effect of limonene nano-emulsion was not only restricted to Gram-negative bacteria (*E. coli*) but a similar effect was also observed with Gram-positive isolates (*B. subtilis* and *S. aureus*)[26]. A similar effect of limonene nano-emulsion was also observed as limonene nano-emulsion altered the cell membranes of *E. coli*, *S. aureus*, and *B. subtilis* which lead to the outflow of intracellular substances from the treated bacterial cells of all three isolates[27]. Moreover, deformed and incomplete shapes of the bacterial cells (*E. coli*, *S. aureus*, and *B. subtilis*) were also scrutinized by scanning electron microscopy due to an increase in the permeabilization of the cells which leads to disruption of membrane integrity by the treatment of limonene nano-emulsion[28].

It is evident by above-mentioned studies that limonene causes damage to the cell membrane of Gram-positive as well as Gram-negative bacterial cells which initiates the leakage of intracellular materials and ultimately leads to cell death. However, the efficacy of limonene or any other antibacterial agent may differ in Gram-positive and Gram-negative cells. The cytoplasmic membrane is known as the primary target in Gram-positive bacterial cells while in Gram-negative bacteria outer membrane is known as the primary target for antibacterial agents[29]. Gram-negative cells are more efficient than Gram-positive bacterial cells in maintaining their membrane homeostasis, which is why there is a difference between antimicrobial agents towards the bactericidal activity[30]. It was also explained that the outer membrane of Gram-negative bacteria, which is composed of lipopolysaccharide molecules establishs a hydrophilic permeability barrier that protects against the effect of highly hydrophobic drugs[31]. This also explains the reason for the low sensitivity of Gram-negative bacterial cells to the lethal effects of lipophilic monoterpenes like limonene.

Most of the studies mentioned above-observed cell membrane damage and alteration in the membrane permeability as the mechanism of action of limonene. However, the precise mechanism of limonene as an antibacterial drug is largely unknown as the series of events which leads to the cell death by the action of limonene are not studied. In a recent study, the effects of limonene on protein expressions related to respiratory chain complex in *L. monocytogenes* were studied. Limonene treatment to bacterial cells was observed to down-regulate different respiratory chain-related complexes[32]. Moreover, additional studies are required to decipher the exact molecular mechanisms of limonene against various other pathogenic Gram-positive or Gram-negative bacteria.

Antifungal

Limonene is also known for its antifungal properties. It has been observed that limonene is effective against various yeasts and molds which are known as the major contaminant of food products (pudding), dairy (yogurt, cheese, and dairy caramels), fruits (Wine grapes, satsumas, apples, strawberries), grains (wheat flour, corn, peanuts, coffee, cocoa powder, cereal), flavored water and chocolate[88]. Some of the yeasts are known to cause contamination in other food products such as meat, vegetables, nonalcoholic beverages, alcoholic beverages, and bakery products[89]. While some of the fungal species are known to be pathogenic to humans. Antifungal properties of limonene have been explored against various fungal species. Various antifungal properties of limonene are summarized in Table 2.

The antifungal mechanism of action of limonene was also studied against few fungal isolates. Treatment of limonene to yeast cells arise

Table 2. Antifungal Activity of Limonene

S. No.	Study	Concentration	Effective against	References
	Antifungal activity of Limonene used in pure form			
1	Antifungal action of two enantiomers of limonene	Pure	*M. gypseum, A. niger, A. flavus, T. rubrum, S. schenckii, C. albicans AI* and *C. albicans*	[38]
2	Antifungal action of limonene against clinical isolates	Pure	*C. albicans*	[40]
3	Antifungal action of limonene against some fungal isolates and dermatomycetes	Pure	*C. albicans, A. niger, A. ochraceus, A. versicolor, A. flavus, A. terreus, A. alternate, P. ochrochloron, P. funiculosum, C. cladosporioides, T. viride, F. tricinctum, P. helianthi, M. canis, E. floccosum, T. rubrum, T. mentagrophytes* and *T. tonsurans*	[41]
4	Antifungal action of limonene and 1,8-cineol alone and in combination	Pure (97%)	*Cryptococcus neoformans*	[58]
5	Inhibitory effect of cyclic terpenes on *Fusarium verticillioides*	Pure	*Fusarium verticillioides*	[94]
6	Antifungal action of Limonene against *Trichophyton rubrum*	Pure	*Trichophyton rubrum*	[95]
7	Antifungal action of limonene against some clinical isolates and dermatomycetes	Pure	*C. albicans, C. parapsilosis, R. rubra, A. niger, P. tardum, C. krusei, E. floccosum, A. fumigatus* and *P. chrysogenum*	[96]
8	Antimicrobial action of limonene against fruit juice spoiling micro flora	Pure	*S. bayanus, P. membranifaciens,* and *R. bacarum*	[43]
9	Antifungal action of d-limonene on toxigenic strains of *Aspergillus*	Pure	*A. flavus*	[97]
10	Antifungal action of limonene used as nanoemulsion	Pure	*Saccharomyces cerevisiae*	[61]
11	The Effects of limonene on Some Spoilage Fungi	Pure	*C. albicans, Penicillium* sp., *Aspergillus* sp. and *A. niger*	[98]
12	Structure alteration of *A. flavus* and *A. parasiticus* by limonene	Pure	*A. flavus* and *A. parasiticus*	[99]
13	Anti yeast activities of limonene in growth medium, fruit juices and milk	Pure	*Saccharomyces cerevisiae*	[100]
14	Antifungal action of limonene	Pure	*C. albicans* and *C. parapsilosis*	[45]
15	Antifungal action of limonene against dermatomycetes	Pure	*T. mentagrophytes, T. rubrum* and *T. tonsurans*	[101]

Table 2. Cont...

S. No.	Study	Concentration	Effective against	References
16	Antifungal and inhibitory effects of DL-limonene on some yeasts	Pure	14 yeast strains	[102]
17	Effect of limonene on *Saccharomyces cerevisiae*	93%	Saccharomyces cerevisiae	[90]
18	Antimicrobial action of limonene from *Wedelia prostrata*	Pure	*H. anomala, S. cerevisiae, A. niger, C. globosum, M. racemosus* and *M. anka*	[47]
19	Response of *Saccharomyces cerevisiae* to D-limonene-induced oxidative stress	Pure	*Saccharomyces cerevisiae*	[91]
20	Antifungal action of limonene from genus Citrus	Pure	*Aspergillus niger, A. flavus* and *A. carbonarius*	[50]
21	Effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion	Pure	*Saccharomyces cerevisiae*	[26]
22	Antifungal action of limonene from *Mentha* L. essential oils	Pure	*A. alternate, A. solani, A. flavus, A. niger, F. solani* and *R. solani*	[103]
23	Improving antimicrobial action of D-limonene using a novel organogel-based nanoemulsion	Pure	*Saccharomyces cerevisiae*	[28]
24	Antifungal action of limonene against biofilm forming pathogenic fungi	Pure	*C. albicans, C. parapsilosis, A. fumigatus, A. terreus* and *S. apiospermum*	[55]
25	Antimicrobial action of limonene	Pure (97%)	*C. albicans* and *A. niger*	[104]
Antibacterial activity of essential oils containing Limonene				
26	Antifungal action of the essential oils of *Cotinus coggygria*	39.2 and 47% (Major)	*A. niger, A. ochraceus, A. versicolor, A. flavus, A. fumigatus, P. ochrochloron , P. funiculosum, T. viride, C.*Albicans and *T. mentagrophytes.*	[105]
27	Antimicrobial action of the essential oil of *Pimpinella puberula* (DC.) Boiss.	21.7-82.4% (Major)	*C. albicans*	[66]
28	Antifungal action of *Citrus reticulata* Blanco essential oil	46.7% (Major)	*Alternaria alternata, Rhizoctonia solani, Curvularia lunata, Fusarium oxysporum* and *Helminthosporium oryzae*	[106]
29	Biological activities of two Citrus species	75.56 and 92.48% (Major)	*S. cerevisiae* and *C. albicans*	[107]
30	Antimicrobial action of Turkish citrus peel oils	36.8 to 94.1% (Major)	*C. albicans, K. fragilis , R. rubra , D. hansenii* and *H. guilliermondii*	[108]

Table 2. Cont...

S. No.	Study	Concentration	Effective against	References
31	Antifungal action of plant essential oils against *Malassezia furfur*	51.07% (Major)	*Malassezia furfur*	[109]
32	Antimicrobial action of essential oils of *Aloysia triphylla*	Major	*C. albicans Hansenula* sp and *Rhodotorula* sp	[110]
33	Antifungal activities of volatile extracts from fresh leaves of *Crassocephalum rubens* against food borne pathogens	48.8% (Major)	*C. albicans*	[68]
34	Antimicrobial action of essential oil of *Citrus limetta* var	95.98% (Major)	*A. niger, A. flavus, A. fumigatus, A. ficuum, F. oxysporum, P. digitatum, F. saloni* and *C. utilis.*	[74]
35	Antifungal action of essential oils in the control of food-borne fungi growth	Major	*A. flavus, A. ochraceus, A. parasiticus, F. verticillioides, E. repens P. corylophilum*	[111]
36	Antimicrobial action of essential oils of Tunisian *Citrus aurantium* L.	87.02%	*C. albicans*	[112]
37	Antifungal efficacy of essential oil from the Egyptian sweet orange peel	86.75% (Major)	*A. flavus,* and *A. paraciticus*	[113]
38	Antimicrobial action of Bergamot Oil	40% (Major)	*Trichophyton verrucosum*	[114]
39	Antimicrobial action of essential oil of *Ammodaucus leucotrichus* Coss. & Dur. seeds	*23.89%* (second highest)	*C. albicans,S. cerevisiae, A. flavus* and *P. escpansum*	[75]
40	Antimicrobial activities of leaves and fruit peels of *Citrus sinensis* Linn.	30.1% (second highest)	*C. albicans* and *A. niger*	[76]
41	Antimicrobial action of Leaf, Ripe and Unripe Peel of Bitter Orange (*Citrus aurantium*) Essential Oils	Major	*Saccharomyces cerevisiae*	[115]
42	Antifungal action of essential oils from *Protium heptaphyllum* against *Candida* spp.	36.01 (Major)	16 species of Candida	[116]
43	Antimicrobial action of *Citrus reticulata* Blanco Cultivar Murcott	13.9 to 93.71% (Major)	*A. fumigates, C. albicans* and *S. cerevisiae*	[117]
44	Antimicrobial action of *Citrus medica* L. var proper leaf essential oil against skin pathogens	11.54% (third highest)	*C. albicans*	[83]
45	Antimicrobial action of *Citrus lemon* essential oil	39.4% (Major)	*A. niger, A. flavus, A. nidulans, A. fumigates, F. graminearum, F.oxysporum, F. culmorum* and *A. alternata*	[118]

a compensatory response in the form of over-expression of various genes (i.e., ROM1, RLM1, PIR3, CTT1, YGP1, MLP1, PST1, CWP1) which are involved in cell wall integrity signaling pathway. It was concluded that limonene treatment leads to cell wall deterioration in the yeast cells. The fact of alteration in structure and function of yeast which further affects the cytokinesis due to limonene was established[90]. Limonene was also observed to induce the accumulation of reactive oxygen species (ROS) in fungal isolates and leads to cell death[91]. Scanning electron microscopic analysis of fungal isolates after the treatment of limonene showed the deformation and distortion of the fungal cells which further leads to the leakage of intracellular material due to damaged cell membrane[26,28]. Mechanism of antifungal action of limonene was also studied against *Candida albicans* in which treatment of limonene cause alteration in membrane permeabilization of up to 82-88% of the cells[55]. Mechanism of action of limonene against *Candida* sp. involved in intra-vaginal infections was also examined. Results of the study showed the protective role of limonene against Vulvovaginal candidiasis causing fungal isolates, as a low fungal burden in mice was observed after the treatment with limonene in comparison to untreated mice. Electron microscopy also revealed that limonene treatment caused dramatic structural changes in fungal cells including cell wall rupture[92]. Limonene is also found to inhibit the intracellular and extracellular enzymes such as cellulase and pectin methyl esterase present in some fungal isolates such as *A. niger*, *P. digitatum*, *F. oxysporium* and *R. solani*[93].

All the above-mentioned studies explained a few of the antifungal mechanisms of action of limonene against various fungal isolates. However, exact mechanisms of the anti-fungal actions of limonene are not yet unrevealed hence; further studies are required to explore the antifungal mechanisms of limonene.

Anti-biofilm production property of Limonene

Resistance in microorganisms against various antimicrobial agents has become the main concern in the medical industry nowadays. The formation of biofilm by microorganisms is one of the mechanisms to acquire resistance against antimicrobial agents. Hence, it is important to find alternative therapeutic agents with anti-biofilm properties. Many plant-based compounds are being examined for their therapeutic properties of which only a few are reported to exhibit anti-biofilm activity[119]. Limonene is one the plant-based compounds which possess anti-biofilm property. The anti-biofilm property of limonene is also investigated and proved in various studies by many researchers. The anti-biofilm property of limonene was examined against *B. cereus*, *E. coli*, *P. anomala* and *P. putida* and significant inhibition of biofilm of *B. cereus*, *E. coli*, and *P. anomala* by limonene was observed[48]. About 75-95% biofilm inhibition was observed against *S. pyogenes*, *S. mutans*, and *S. mitis* at 400µg/ml concentration of limonene[120]. Limonene was also found to reduce the biofilm mass production up to 90% after 8h of incubation at the concentration of 2000µl/L in different strains of *S. aureus*[121]. In another study, effective biofilm inhibition by limonene against *P. aeruginosa*, *C. albicans*, and *C. parapsilosis* was also observed[55]. *In silico* determination of anti-biofilm property of limonene against *S. mutans* showed that limonene can act as a good candidate for the inhibition of development in biofilm formation[122].

Concluding Remarks and Future Perspectives

As discussed above, limonene possesses antimicrobial properties such as antibacterial, antifungal, and antiviral. Limonene was also found to hinder biofilm formation and most importantly limonene also shows the inhibitory action against COVID-19. Limonene is suggested to be safe as it is derived from natural sources e.g. citrus plants. Hence, the use of limonene in the future for therapeutic purposes in clinical settings may be considered. Moreover, researches on combinatorial studies of limonene with other drugs are also inevitable to achieve better outcomes in breaking the therapeutic resistance of microbes. Delineation of the mechanistic approach of limonene towards antimicrobial activities is also desirable. Therefore, it may be suggested that there is still a wide lacuna of knowledge to be acquired to venture limonene as a future potential drug molecule.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Not applicable.

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