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Original Research Paper

Limonene extraction from the zest of *Citrus sinensis, Citrus limon*, *Vitis vinifera* and evaluation of its antimicrobial activity

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ABSTRACT

Citrus rinds contain essential oils. One of the major constituents of the essential oils in the zest of different fruits like *Citrus sinensis, C. limon,* and *Vitis vinifera* is limonene. In this research, limonene was extracted by hydro-distillation method using Clevenger set up and its antimicrobial activity against certain bacterial and fungal strains was determined by using Kirby Bauer's disc diffusion method. The primary antimicrobial screening of limonene without dilution exhibited a zone of inhibition (mm) comparable to Ampicillin (20mg/ml) and Amphotericin B (20mg/ml). The effect of pure limonene against all strains used was high as compared to the isolated samples. The MIC values also showed an expected decrease in the zone of inhibition from 1:2 to 1:8 dilutions. Based on this study, the cost-effective isolation of limonene and other essential oils is quite possible.

Keywords: Anti-microbial citrus, hydro-distillation and limonene

INTRODUCTION

Citrus fruits are generally cultivated throughout the world. A substantial load of waste is produced after their processing. An estimated amount of Citrus mash (CM) generated in South Korea was reported to be more than sixty thousand tons. Not only the edible part but also the citrus peels are well known for their phytochemical, polyphenolic, vitamin, and essential oil contents (Mahato *et al*., 2017). One of the major components of citrus waste is the limonene. In citrus, the outer skin known as zest contains the majority of the Limonene, whereas, traces of limonene can be isolated from the inner white kernel. Limonene is a colourless cyclic monoterpene with chemical formula C10H16 having 1-methyl-4(prop-1-en-2-yl) cyclohex-1-ene as its IUPAC name and commonly used in food and pharmaceutical industries (Atti-Santos *et al*., 2005). It derives its name from the lemon as it can be derived from lemon peel. Limonene in its two optical forms is not only found in the citrus fruits but is known to exist in about 300 plant essential oils (Bacanli *et al*., 2015). This ten carbon compound is known to be volatile in nature and is also prone to oxidation. This oxidation generally occurs at the time of extraction and packaging. Limonene has found a wide range of applications as a flavoring and fragrance agent in many products, such as perfumes, beverages, detergents and soaps (Erasto and Viljoen, 2008). Besides this, limonene is well known for its insecticidal properties as it can penetrate the insect body through the respiratory system (fumigation), the cuticle (contact effect), or the digestive system (ingestion effect)(Prates *et al*., 1998) .

Limonene has a characteristic citrus odor and is colorless with a molar mass of 136.24g/mol. Since it has a chiral center with four functional groups around so, it exists in two isomeric forms I, e d-limonene and l- limonene. The d-limonene is common in lime, lemon, orange, tangerine, etc. It has a density of about 84kg/m3 which is lesser than the water I, e 997kg/m3 (Erasto and Viljoen, 2008).

The presence of this 10 carbon compound is also attributed to various plant genera like *Lippia* (frog fruit) and *Artemisia* (Mugwort, Sagewort). This optically active compound is generally found in its d-form. However, l-form is also found in *Pinus* and *Menthe* species. Limonene has been found to exhibit herbicide and antifeedant properties and acts as an

attractant for pollinators. This compound with its two isoprene units has tremendous antioxidant properties as it saturates the pulmonary membrane and gives protection against endogenous and exogenous oxidant agents like ozone (Erasto and Viljoen, 2008). Limonene can generally be extracted by various methods like steam distillation, cold press, solvent extraction, and hydro-distillation using the Clevenger system.

The emergence of drug resistant bacterial and fungal species poses a serious global public health concern. Hence, it is imperative to search for novel compounds to contain these pathogens. In this regard phytochemicals can be very useful. Hence, in this study antibacterial and antifungal properties of limonene extracted from *Citrus sinensis, C. limon,* and *Vitis vinifera* were evaluated.

MATERIAL AND METHODS

Sample collectiona and extraction:

The samples of the known variety of *C. sinensis, C. limon,* and *V. vinifera* were collected from the local street vendor in the commonly used polythene bag. The samples were washed initially with tap water and then with distilled water to make the outer surface sterilized (Shaw *et al*., 1997).

The fresh orange (1000g), Lemon (500g), and grape (500g) after surface sterilization were gently pressed against the grater to remove the zest without removing any part of the white flesh. The peel of *V. vinifera* was obtained manually. Then the peels of *V. vinifera* and zest of *C. sinensis* and *C. limon* were transferred to round bottom flasks (RBF). After that water was added into the RBF along with porcelain chips to prevent splashing of the zest and peels to the neck and allow smooth boiling (Gelb *et al*., 1995; Malko and Wróblewska, 2016). The distillation process was proceeded at a temperature of 70°C. The distillate was collected after one and a half hours and then continuing the process again. The distillate was left undisturbed for about 20 minutes and then removed carefully using a pipette.

Limonene isolation and detection

The crude essential oil rich in limonene isolated from zest of *Citrus sinensis, Citrus limon,* and *Vitis vinifera* were detected by comparing the odour of the pure limonene with that of the isolated samples. Furthermore, the detection of crude limonene was performed by comparing the pH with that of pure limonene (Lopez-sanchez and Pagliaro, 2014). For the detection of the crude limonene from the zest of *C. sinensis* (OIL)*, C. limon* (LIL), and *V. vinifera* (GIL) biochemical assays such as Iodine test and Bromine test as described by Wang and Weller, 2006, and Asbahani *et al*., 2015, respectively.

Culture revivaland antimicrobial activity determination

The strains which include gram-positive (*B.subtilis, M.luteus, and S. aureus*), gram-negative (*E.coli, K.pneumoniae, A.hydrophila, and P.pituda*), and fungal (*C.albicans, C.parapsilosis and S.cerevisiae*) were obtained from microbial type culture collection (MTCC) (Lucchesi *et al.*, 2004)**.** All the bacterial and fungal cultures were initially revived in the nutrient broth and yeast extract peptone dextrose (YEPD) broth, respectively and growth was checked as per the method described by Bhattacharya *et al*., (2014).

After the revival of microbes the antimicrobial activity of the crude essential oils rich in limonene was determined by using Kirby Bauer's disc diffusion method (Akhtar *et al*., 2017a; Akhtar *et al*., 2017b; Choudhury *et al*., 2017). In brief the microbes were grown overnight at 37°C and 28°C in Nutrient broth and YEPD broth for bacterial and fungal species respectively. Then 100 µl of the culture were spread on Nutrient agar and YEPD agar plates. Then, sterile filter discs containing different concentration of the essential oils rich in limonene extracted from zest of *C. sinensis, C. limon* and *V. vinifera* were placed on the plates. The plates were further incubated at 37°C for bacterial species and 28°C for fungal species. After the incubation, the zone of inhibition was measured. Ampicillin was used as control for bacterial species and Amphotericin B was used as control for fungal species.

RESULT AND DISCUSSION

Isolatoion and detection of limonene

Distillate obtained after Hydro-distillation is given in the table 1. The pH of the crude essential oils was almost the same as that of pure limonene. The color change during the biochemical assays such as Iodine test and Bromine water test also confirmed the presence of limonene in the crude essential oils (Figure 1 and Figure 2). The limonene content in orange zest, limonene zest, and grape peels were 2.0 mL, 2.7mL and 3.46mL for every 100 g of samples respectively.

Table 1. Distillate obtained after Hydro-distillation

Fruit name	Zest weight (g)	Solvent added (ml)	Tempe-rature (°C)	Initial distillate (ml)	Limonene volume (ml)
Orange	150	150	70°C	13	3
Lemon	90	200	70°C	10	2.5
Grape	130	150	70°C	16	4.5

Microbial susceptibility

The maximum anti-microbial activity was shown by the pure limonene (97%) for all the tested strains. Among the ten strains, the maximum effect of pure limonene was found in the case of *B. subtilis* with a zone of inhibition (ZOI) measuring about 12mm. The minimum effect was revealed against *C. albicans* with ZOI measuring about 3mm (Karr, 1989). On comparing the results of three crude essential oil isolates the maximum effect was shown by the OIL and LIL against *K. pneumoniae* (7mm) and *S. aureus* (8mm) respectively. The crude essential oil rich limonene isolated from the grape peels showed good positive effects against *E.coli* (0.7mm) and *B. subtilis* (0.5mm) while it showed ZOI in the range of 1mm-3mm against all other strains (Figure 3, 4, 5) (Bhattacharya *et al*., 2014) . However, GOl, was found to be ineffective against all the fungal strains. So, pure limonene showed the maximum anti-microbial activity in comparison to the three isolates, whereas, the OIL and LIL showed maximum effect against gram positive bacteria than those of gram negative bacteria. The effects shown by the limonene are given below (Table 2):

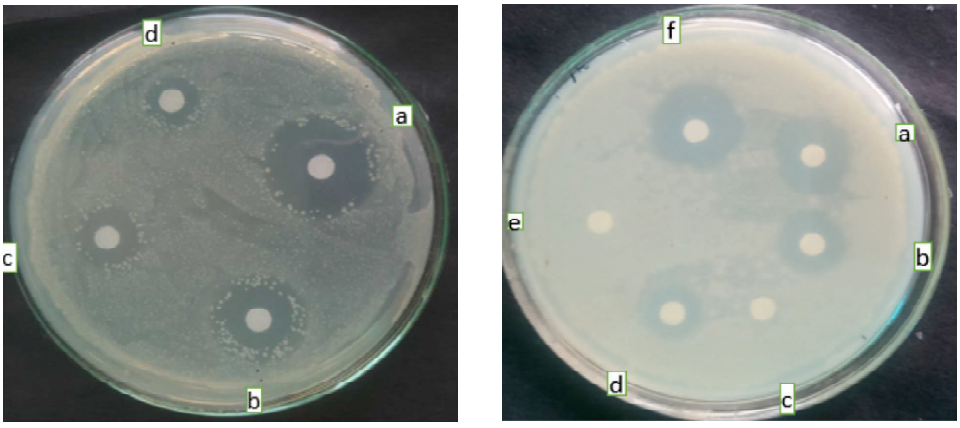

Figure 1. The antimicrobial activity of (i) pure limonene (PL), (ii) crude essential oil isolated from C. limon (LIL), against *E. coli* at different concentration- (a). 1:2 (b). 1:4 (c). 1:6 (d). 1:8

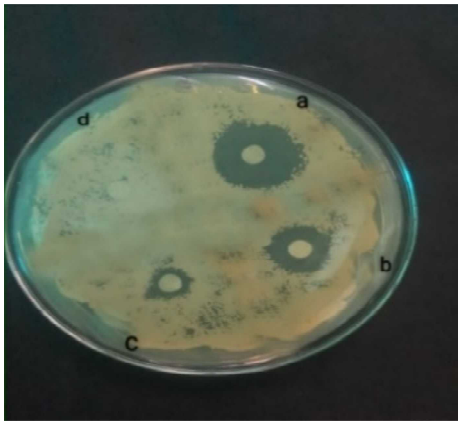

Figure 2. Antifungal activity of essential oils against *C. parapsilosis* (a). PL (b). OIL (c). LIL (d) GIL

Table 2. Anti-microbial activity of essential oils against different bacteria and fungi

Organisms	Organism type	MTCC number	Incubation time (h)	Pure limonene	Zone of inhibition (mm)		
					Orange isolate	Lemon isolate	Grape isolate
M. luteus	GPB	106	24	6	6	5	3
P. putida	GNB	102	27	5	6	4	3
A. hydrophila	GNB	1739	26	4	3	4	3
E.coli	GNB	739	25	6	2	8	7
S. aureus	GPB	3160	24	7	5	6	2
B. subtilis	GPB	121	24	10	4	2	5
K. pneumoniae	GNB	7028	27	7	6	5	3
C. albicans	Fungi	183	30	3	1	0.00	0.00
C. parapsilosis	Fungi	998	30	5	4	3	0.00
S. cerevisae	Fungi	36	30	5	4	2	1

Microbiostatic/microbicidal effect of limonene with different dilutions:

All those bacteria showing a zone of inhibition equal to 4mm or more were tested for determining the microbiostatic/microbicidal of a particular type of limonene. On the other hand, those with ZOI below 4mm were not tested (-). There was a continuous decrease in the ZOI of inhibition from less diluted sample to a more diluted sample (Figure 4 and 5). Besides the continuous decline in the ZOI, there were some unusual results shown by the *P.putida* for 1:2 and 1:4 LIL. Similar uncommon results were shown by *E. coli* and *B. subtilis* (Bold letters). Similar type of findings were reported in previous studies against *E. coli, P. putida,* and *S. aureus* (Akhtar *et al*., 2017a, 2017b; Bilal Ahmad *et al*., 2020). *C. parapsilosis* and *S. cerevisae* showing ZOI equal to 4mm or more for OIL and PL respectively were tested using different dilutions of these two samples. *C. albicans* was not tested because it did show susceptibility without dilution. There was a continuous decrease in the ZOI from less diluted sample to more diluted samples. The summary of microbiostatic/microbiocidal effect of crude essential oils rich in limonene with different dilutions is given in Table 3.

Table 3. Microbio-static/Microbicidal effect of limonene with different dilutions

ZOI by ampicillin, PL, OIL, LIL and GIL (mm)																	
Orga-nism MTCC	+ve Control 25 mg/ml	Pure limonene				Orange isolated limonene				Lemon isolated limonene				Grape isolated limonene			
		1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8	1:2	1:4	1:6	1:8
106	7	4	2	0	0	4	3	0	0	3	2	0	0	-	-	-	-
102	5	4	3	1	0	3	2	1	0	3	0	2	0	-	-	-	-
1739	6	3	2	1	1	-	-	-	-	0	0	0	0	-	-	-	-
739	7	6	4	3	2	-	-	-	-	5	4	0	2	4	3	1	0
3160	7	3	2	1	1	4	3	2	1	3	1	1	0	-	-	-	-
121	5	3	4	2	1	4	3	2	1	-	-	-	-	4	2	1	1
7028	6	3	1	0	0	2	1	0	0	2	1	0	0	-	-	-	-
183	1	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
998	5	0	0	0	0	-	-	-	-	0	0	0	0	-	-	-	-
36	5	0	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-

CONCLUSION

In the study crude essential oil rich in limonene were extracted from *Citrus sinensis, Citrus limon,* and *Vitis vinifera* by hydro-distillation with Clevenger set-up. The detection of limonene in these crude essential oils were confirmed by different biochemical assays. The isolated crude essential oil rich in limonene showed potential antimicrobial activity against different Gram-positive and Gram-negative bacteria as well as also three fungal species. This study shows that the limonene rich essential oils of *C. sinensis, C. limon* and *V. vinifera* has the potential to be used in drug and food industry to control various pathogens.

Conflict of interest: The authors declare no conflict of interest.

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