Research Article

Quantitative Analysis and Antimicrobial Efficacy of Juice and their Extracts from *Citrus Sps.* Against Selected Microorganisms

Naynika Patel*, JSS Mohan
B.R.D. School of Biosciences, Sardar Patel University, Vallabh Vidhyanagar, 388 120, Gujarat, India.
***Corresponding author's E-mail:** naynikapatel24179@gmail.com

Received: 06-03-2024; **Revised:** 23-04-2024; **Accepted:** 30-04-2024; **Published on:** 15-05-2024.

ABSTRACT

Antimicrobial efficiency of *Citrus limon* Burn. and *Citrus medica* L. different juice concentration and juice extract were examined by solvent fractionation partition extraction using n-Hexane and ethyl acetate as solvents and tested against six gram positive and six gram negative bacteria and five fungi by agar well diffusion method using positive control. The *Citrus limon* exhibited antimicrobial activities with zones of inhibition ranging from 7 to 45mm, 7 to 20mm, 7 to 21mm and 7 to 18mm for fresh juice and Juice: DMSO (1:1), n-Hexane, Ethyl acetate and D/W extracts respectively. Similarly *Citrus medica* displayed antimicrobial activities with zones of inhibition ranging from 7 to 27mm, 7 to 24mm, 7 to 25mm and 7to 19mm for fresh juice, Juice: DMSO (1:1) and n-Hexane, Ethyl acetate and D/W extracts respectively. MIC and MBC value ranging from 0.125mg/ml to 4mg/ml for different juice concentration and 4mg/ml to >8mg/ml found in different juice extract. n-Hexane (16mm) and ethyl acetate (18mm) juice extract of *Citrus medica* exhibited good zone of inhibition against *Aspergillus niger* and both *Citrus sps*. showed presences of phenolic content range from (3.81mg/ml-5.02mg/ml) and flavonoid content (3.09mg/ml-7.28mg/ml). These results encourage the identification of active substances which could be used as lead(s) molecules in development of new antimicrobial drugs.

Keywords: Antimicrobial, Extract, MIC, MBC, Phenolic and Flavonoid.

INTRODUCTION

India has a rich tradition in use of medicinal plants to develop drugs. According to world health organization (WHO), any plant which contain substances that can be used for therapeutic purpose. Pharmacological industries have produced a number of new antibiotics in the last three decades; resistance to these drugs by microorganisms has increased currently due to use to treat a variety of human disease. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [1]. The clinical efficiency of many antibiotics in existence is being treated by the emergence of multi drug-resistant pathogen[2]. To overcome this problem of resistance of bacteria, researchers concentrate their study to find out new drug from medicinal plants. Several microorganisms derived antibiotics are currently used in develop new drugs either synthetic or natural, for a long period of time, plant have a valuable source of natural products for maintaining human health. Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being. Their role is two fold in the development of new drugs: they may become the base for the development of a medicine, a natural blue print for the development of new drugs or; a phytomedicine to be used for the treatment of diseases [3]. Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world[4]. Medicinal plant would be the best source to obtain a variety of drugs as the phytochemical are more specific. Phytochemical offer unique platform for structural diversity & biological functionality which is indispensable for drug discovery. Therefore, such plants should be investigated to better understand their properties, safety and efficiency.

Citrus is one of the most important commercial fruit crops grown in all continents of the world. *Citrus limon* Burn. and *Citrus medica* L. are medicinal plant belongs to family Rutaceae. It is cultivated mainly due to its anticancer activities and the antimicrobial potential in juice extract [5]. Many studies have reported antioxidant and antibacterial effect of juice and edible parts *Citrus sps* of different varieties [6]. The fruit juices exhibit significant antibacterial effect, the bioactivity being associated with mineral content and biologically active constituents. Hence these fruit juices with the property of bioavailability and the retention of certain minerals by polyphenolic compounds can be recommended for their use as an alternative anti-infective agent in natural medicine for treatment of infectious diseases[7]. For long period of time, medicinally important fruit juices have been a valuable source of natural product for maintaining human health. The use of fruit juices and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments [7,8].Some studies have indicated that foods containing high amounts of flavonoids may reduce the risk of heart diseases, due to their antioxidant properties [9,10]. Studies of the antimicrobial activities of flavonoids have become important because of the increasing occurrence of opportunistic systemic mycosis, as well as the rising prevalence of drug resistance in human pathogenic bacteria[11].Flavonoids can function as direct antioxidants and free radical scavengers, and have the

capacity to modulate enzymatic activities and inhibit cell proliferation [12]. In plants, they appear to play a defensive role against invading pathogens, including bacteria, fungi and viruses[13]. Flavonoids are generally present in glycosylated forms in plants, and the sugar moiety is an important factor determining their bioavailability. Citrus fruit juice is known to potent antimicrobial agents against bacteria and fungi. These citrus fruits are rich sources of flavonones and many polymethoxylated flavones which are very rare in other plants [7,14].

This study was aimed for the assessment of phytochemical constituents and antimicrobial activity of *Citrus limon* and *Citrus medica*.

MATERIALS AND METHODS

Collection of plant materials

Two plant species belonging to family Rutaceae were collected in the form of fruit from different localities of Gujarat (Table-1). All the specimens were identified by referring "Flora of Gujarat state" [15] and confirmed by Dr. A.S. Reddy (Plant Taxonomist), Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Gujarat.

Extract preparation:

Solvent fractionation partition

Solvent fractionation was used as a preliminary separation to simplify complex extracts with promising activity by fractionating chemical compounds into broad groups based on their solubility. The crude juices were partially purified by solvent-solvent extraction method. Crude juice plant extracts were mixed in 100% ethyl acetate and partitioned with hexane (3x50ml) in glass separating funnel. All fractions were pooled individual n-Hexane fraction, ethyl acetate fraction and water fraction concentrated with the solvent recovering assembly. Concentrated fraction was dried and checked for presence of antimicrobial activity and phytochemical analysis [16].

Microbial pure cultures were obtained from MTCC (Microbial type culture collection, Chandigarh), ATCC (American type culture collection, Manassas, Virginia) and NCTC (National collection of type culture). The bacterial and fungal cultures were grown on nutrient agar medium (Hi Media, pH 7.4) at 37^{o}C and potato dextrose agar medium (Hi Media, pH 5.6) at 27^{o}C respectively. Both the cultures were maintained at 4^{o}C.

Table 1: List of selected medicinal plants from different localities of Gujarat.

Plant name	Family	Plant part	Location
***Citrus medica* L.**	Rutaceae	Fruit	Rajnagar
***Citrus limon* Burn**	Rutaceae	Fruit	Karamsad

Selected microorganisms (Table 2)

In the present study, the following six Gram-positive, six Gram-negative bacteria and five fungi were selected.

Types of microorganism	Microorganism strains	Causes
Gram positive	*Bacillus cereus* (ATCC 11778)	Food poisoning, vomiting, Diarrhoea,
	Bacillus subtilis (ATCC 6051)	Food poisoning
	Staphylococcus aureus (Isolated)	Wound infection, Pneumonia
	Staphylococcus epidermidis (ATCC 155)	Infection of prosthetic medical device
	Micrococcus luteus (ATCC 4698)	Septic shock, septic arthritis
	Enterococcus faecalis (Isolated)	Carcinoma, dysplasia, inflammatory bowel disease
Gram negative	*Escherichia coli* (ATCC 25922)	Bloody diarrhoea, kidney diseases.
	Salmonella typhi (NCTC8394)	Typhoid, enteric fever
	Salmonella paratyphi (MTCC 735*)*	Paratyphoid fever and typhoid
	Pseudomonas aeruginosa (ATCC 25668)	Septicaemia, pneumonia, dermatitis
	Klebsiella pneumoniae (ATCC 15380)	Pneumonia, flu, chill and cough
	Serratia marcescens (Isolated)	Bacteremia, urinary and respiratory infection
Fungi	*Aspergillus niger* (MTCC40211)	Skin and ear diseases
	Candida albicans (MTCC 183)	Skin and gastrointestinal infection
	Trichoderma harzianum (Isolated)	Beneficial antagonistic effect
	Fusarium oxysporum (Isolated)	Wilting of leaves
	Aspergillus flavus (MTCC4613)	Aspergillosis

Antimicrobial assay

In the present study, the antimicrobial activity of crude juice and juice extract in different solvents (ethylacetate and n-Hexane) were screened by agar well diffusion method [17].

Antibacterial activity

An inoculum size of 1×10 CFU/ml of bacteria which compared with 0.5 McFarland turbidity[18] standard in a refrigerator for 30 minutes for pre-diffusion of plant extract and turbidity standards was used. Each extract of 100 µl

(stock solution 100 mg/ml) was added in a previously marked sterile nutrient agar petriplates and the wells were punched with sterile cork borer and filled with each plant extract. Plates were incubated at 37°C for 24 hours. After incubation all the plates were examined and zone of inhibition (excluding well diameter in mm) was measured as a property of antimicrobial activity. Antibiotic such as Ciprofloxacin and Doxycycline (20µg/ml) as a positive control and 100% DMSO as a negative controls.

Antifungal activity

The fungal spores were harvested in sterile distilled water from seven days old culture for determination of antifungal activity. The fungal spores count was counted using haemocytometer under aseptic condition, in laminar air flow the potato dextrose agar medium pour into presterilized petriplate and inoculated by fungal strain respectively and kept for 10-15 minutes for solidifying. Each extract of 100 µl (stock solution 100 mg/ml) was added in a previously marked sterile Potato dextrose agar petriplates and the wells were punched with sterile cork borer and filled with each plant extract. Plates were placed then incubated at 27ºC for 48 hours. After incubation all the plates were examined and zone of inhibition (excluding well diameter in mm) was measured as a property of antifungal activity. Antibiotic such as Fluconazole and Ketacozole (20µg/ml) as a positive control and 100% DMSO as a negative controls.

Minimum inhibitory concentration (MIC):

In the present study, MIC was evaluated by serial broth dilution method[19] for the plant extracts showing more than 7mm zone of inhibition. Density of bacterial suspension was maintained uniformly throughout the work at $1x10^8$ CFU/ml by comparing with 0.5 Mc Farland turbidity standards. 40µl of plant extract from stock solution (100mg/ml) was taken into the first dilution tube and added 960µl of nutrient broth and mixed well. 500µl of solution from first dilution tube was taken and added 500µl of nutrient broth into second tube, this step was repeated 5times and from last tube 500µl solution was discarded. Final volume was made up to 1ml by adding 500µl of test organism in each tube. The MIC was tested in the concentration range between 8mg/ml to 0.250mg/ml. Tubes were incubated at 37°C for 24 hours in an incubator.100µl (0.1%) 2,3,5 – triphenyl tetrazolium chloride solution as a growth indicator was incorporated in each tube to find out the bacterial inhibition and tubes were further incubated for 30 minutes at 37°C. Bacterial growth was visualized when colorless 2, 3, 5-triphenyl tetrazolium chloride was converted into red color formazon in the presence of live bacteria. MIC assay was repeated thrice by using DMSO and nutrient broth as controls.

Minimum bactericidal Concentration (MBC):

To determine the MBC, for each set of test tubes in the MIC determination, l00µl of broth was collected from those tubes which did not show any growth and spreading on sterile nutrient agar plate for any bacterial growth. Plates were incubated at 37°C for 24 hours. After incubation the concentration at which no visible bacterial growth was observed considered as the minimum bactericidal concentration [20].

Quantitative phytochemical analysis of crude extract:

The crude extracts of *Citrus limon* and *Citrus medica* were evaluated for quantitative analysis of phenols and flavonoids by using standard procedures.

Total Phenol estimation:

Reagents:

Folin-Ciocalteu Reagent (FCR) (1:1), 20% sodium carbonate, standard catechol solution (1 mg/ml), working catechol solution (0.1 mg/ml).

Procedure:

Plant extract (0.2 ml) in test tube was taken and added 3 ml distilled water and then added 0.5 ml FCR. After 3min. incubation, 2 ml of 20% Na_2CO_3 solution was added into each tube and mixed thoroughly. Reaction tubes were placed in boiling water bath for exactly 1 min, cooled and the absorbance was measured at 650 nm against a reagent blank using visible spectrophotometer. A standard curve was prepared using different concentrations of catechol. Total phenol was expressed as mg phenol in terms of catechol per gram of fresh tissue [21].

Total Flavonoids estimation:

Reagent:

10% Aluminium chloride, 1M Potassium acetate and working solution Quercetin (1mg/ml).

Procedure:

Aluminum chloride colorimetric method was used with some modification to determine flavonoids content. Add 1 ml of sample plant extract was mixed with 3ml of methanol, 0.2ml of 10% Aluminium chloride, 0.2ml of 1M Potassium acetate and 5.6ml of D/W and remains at room temperature for 30minutes.The absorbance was measured at 420nm.Quercetin was used at standard (1mg/ml). All the tests were performed in triplicates. Flavonoid content was determined from the standard curve and were expressed as Quercetin equivalent (mg/g) of extracted compound [22].

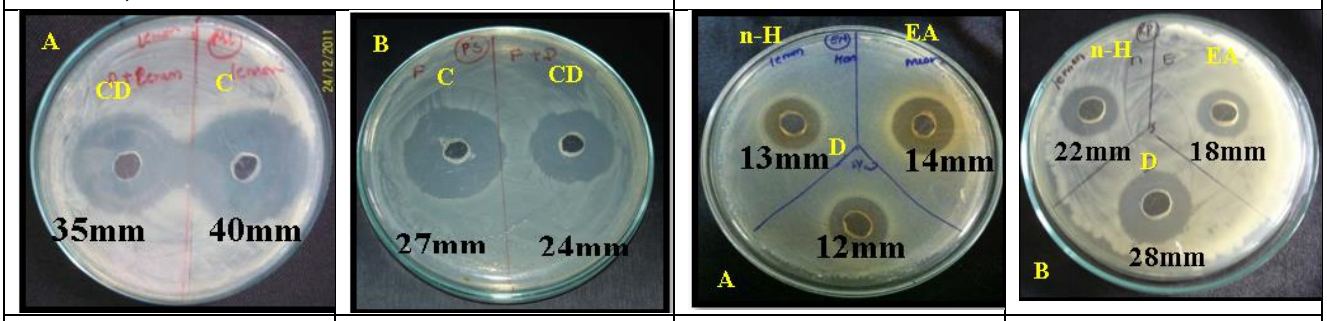
RESULTS AND DISCUSSION

The results of the antimicrobial response shown by juice and their extract from fruit of *Citrus limon* and *Citrus medica* extracts are summarized in (Table 3-8). All the extracts prepared exhibited broad spectrum of antimicrobial activity against selected microorganisms. However, the data indicates that the juice and their extracts prepared in organic solvents consistently displayed better antimicrobial activity along with aqueous extracts. *Citrus limon* fresh juice displayed better activity against BC, SA, EN (30mm), BS, SE, ST (45mm), ML (40mm) (Fig.1-A), EC, SM (29mm), KP (22mm) and PS (23mm) where equal concentration of juice and DMSO(1:1) exhibited better activity against SE(45mm),

ST(44mm), BS(40mm), ML(35mm) (Fig.1A), SP(32mm), BC,EN,(29mm), PS(27mm), SM(28mm), SA,EC(25mm) and KP(24mm). Similarly the crude fresh juice of *Citrus medica* exhibited better activity against all selected bacterial strains PS (27mm) (Fig.1-B), better activity against ST(20mm), EC(19mm), BC,EN,ML,SA,KP (18mm), BS(17mm), EN, SE(16mm), SM and SP (12mm) while the equal concentration of crude juice and DMSO (1:1) better activity against PS(24mm) (Fig.1-B), good activity against SA(16mm) BC, ML,EC,KP and ST (15mm) while moderate activity against SE(14mm), BS,EN(13mm), SM,SP(10mm) similar results was reported [7] that fresh crude Lemon fruit juice produced the highest antimicrobial activity against *Salmonella para.B* and *Shigella sonnei* followed by *E.coli*.

Figure 1 A&B: Antibacterial activity of juice of *Citrus species* against selected tested bacteria (zone of inhibition in mm given in brackets).

Figure 2. A&B: Different extracts of Citrus species fraction showing antibacterial activity against various tested bacteria (zone of inhibition in mm given in brackets)



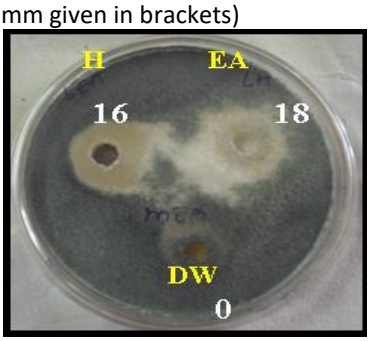
Micrococcus luteus	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	*Klebsiella pneumoniae*

1A– *Citrus limon* juice proportion against *Micrococcus luteus* Crude (40mm) DMSO: Crude (35mm)
1B– *Citrus medica* juice proportion against *Pseudomonas aeruginosa* Crude (27mm) Crude: DMSO (24mm)

2A– *Citrus limon* fraction extract against *Enterococcus faecalis* n-hexane extract (13mm) Ethyl acetate extract (14mm) Distilled water extract (12mm)
2B– *Citrus medica* fraction extract against *Klebsiella pneumoniae* n-hexane extract (22mm) Ethyl acetate extract (18mm) Distilled water extract (28mm)

n-Hexane fraction of *Citrus limon* juice exhibited activity against PS(20mm), EN(13mm) (Fig.2-A), ML(12mm), KP(11mm), BC(10mm), SM(9mm), BS,SA,EC(8mm) and SP(7mm) while ethyl acetate extract found activity against PS(21mm), ML(17mm), EN and SA(14mm) (Fig.2-A), SM(13mm), EC,KP,BS(12mm), BC(11mm), SE and SP(8mm) and water fraction PS(18mm), EN(12mm))(Fig.2-A),SA(11mm), SP(10mm), SM(9 mm) and BC,BS,EC and KP(8mm) and *Citrus medica* n-Hexane fraction of juice exhibited activity against PS(24mm),KP(22mm)(Fig.2-B),EN(19mm), BS(18mm), SA,SE(17mm), ML(16mm), EC,SM(12mm), BC(11mm), ST(9mm) and SP(8mm) while ethyl acetate fraction showed PS(25mm), EN(21mm), KP(18mm) (Fig.2-B), BS(16mm), BC,SA,SM (14mm), SE(12mm) EC,ST(11mm) and SP(10mm) whereas water fraction EN(29mm), KP(28mm) (Fig.2-B), SM(25mm), PS(24mm), BC,SE,EC(23mm) ML(22mm), BS(20mm), SA(17mm), ST(14mm) and SP(8mm). Among the fungal strains, most of the fungi demonstrated good antimicrobial activity; however, *Aspergillus niger, Fusarium oxysporum* and *Tricoderma harzianum* found to be sensitive as compared to the other strains. *Candida albican, Aspergillus parasiticus, Aspergillus flavus*, was the most resistant fungi, showing least activity with all the selected juice and their extracts of *Citrus limon* while *Citrus medica* n-Hexane juice fraction exhibited zone of inhibition (16mm) and Ethyl acetate (18mm) against *Aspergillus niger* (Fig.3) where as [23] reported antimicrobial activity against gram negative and gram-positive bacteria and *C. albicans* from volatile oil of *Citrus reticulata* fruit. All the microorganisms were more sensitive to the n-Hexane, methanol and water fraction.

Figure 3: Different extracts of *Citrus medica* fraction showing antifungal activity against tested fungi (zone of inhibition in mm given in brackets)



Juice fractions against *Aspergillus niger* n-Hexane extract (16mm) Ethyl acetate extract (18mm) Distilled water extract (0mm)

The MIC value of *Citrus limon* fresh juice and equal amount of fresh juice and DMSO(1:1) was observed as 0.125mg/ml against SE,ML, 0.5mg/ml against BS,EC, 1mg/ml against BC,SA,EN,PS,SM, 1mg/ml against PS,SM and 4mg/ml against KP(Table-8) whereas as n-Hexane fraction showed MIC value BC,BS,ML,EC,ST,SP,SM (4mg/ml) and SE,EN(8mg/ml) and KP,PS(>8mg/ml) (Table-6) while MIC in ethyl acetate found mostly BC,BS,EN,ML, SP,ST and SM (4mg/ml) and SE, SA (8mg/ml) (Table 7) and water fraction EC,SE(4mg/ml) BS,SA,KP (8mg/ml) and BC,EN,ML,PS and SM (>8mg/ml)(Table-8).

The MIC value of *Citrus medica* fresh juice, ratio of fresh juice and DMSO (1:1) was observed 0.125mg/ml against BS, SE, ML while 0.5mg/ml against ST, SP and KP whereas 1mg/ml against BC, SA, EN, EC, PS and SM. (Table-8) MIC value in n-Hexane fraction BC,BS,SE,ML,EC,ST, (4mg/ml),

EN(2mg/ml), KP,SM,SP (8mg/ml) and PS(>8mg/ml) (Table-6) while ethyl acetate fraction SE (2 mg/ml) BC,BS,EN,SM (4mg/ml) ML,SA,EC,KP,SP,ST(8mg/ml) and PS(>8mg/ml) (Table-7) whereas water fraction BC,BS,EC (4mg/ml) EN,ML,SE,KP,ST (8mg/ml) and SA,PS,SM,SP (>8mg/ml) (Table-8) similar work reported [7] on antimicrobial activity of *C.limon* and *C. ourantium* fresh juice against enteric pathogen *E.coli, S. paratyphi* and *Shigella somnei* and MIC value was 75% in *E.*coli, 25% *Shigella somnei* and *S.paratyphi*. Minimum bactericidal concentration value of Fresh juice, juice: DMSO (1:1) and juice fractions of *Citrus medica and Citrus limon* demonstrated complete inhibition of microorganism at lower concentration. *Citrus limon* and *Citrus medica* n-hexane inhibit the growth of BC, BS and EC at (4mg/ml) and EN(2mg/ml) in *Citrus medica*(Table 9-11).*Citrus limon* crude juice showed bactericidal activity in BC(2mg/ml), BS(4mg/ml), ML(4mg/ml), SE(8mg/ml), EC(0.5mg/ml) whereas ethyl acetate juice fraction complete inhibited growth in BC,SE(8mg/ml), whereas *Citrus medica* showed bactericidal growth in BC(2mg/ml), BS, ML,SE (4mg/ml) and ethyl acetate fraction in BC &BS (4mg/ml), SE(2mg/ml).*Citrus limon* juice water fraction showed bactericidal activity against BS (8mg/ml), & EC (4mg/ml) whereas *Citrus medica* in BC,BS(8mg/ml) and EC(4mg/ml) similar work done my different research[24] reported different parts of *Citrus aurantifolia* extract produced antimicrobial activity with lower MIC and MBC value; [25] find out the antimicrobial activity of ethanolic, methanolic, ethyl acetate & hot water extract of lemon fruit parts with least concentration of MIC and MBC value against *S.aureus*;[26] investigated the phytochemical composition and antioxidant and antimicrobial activities of different citrus juice concentrates showing lower MIC and highest MBC value.

Table-3: Antibacterial activity in the crude n-Hexane extract of selected *Citrus* species

Plant Name	Part used	Extract	Zone of Inhibition(mm)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	n-H:EA F	10	8	13	12	8	6	8	11	20	9	7	6
Citrus medica	Juice	n-H:EA F	11	18	19	16	17	17	12	22	24	12	8	9
Ciprofloxacin (20 µg/ml)			11	10	12	9	14	11	7	10	9	22	8	14
Doxycycline (20 µg/ml)			14	12	9	8	11	5	15	13	4	20	11	19

Table 4: Antibacterial activity in the crude ethyl acetate extract of selected *Citrus* species

Plant Name	Part used	Extract	Zone of Inhibition (mm)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	n-H:EA F	11	12	14	17	14	8	12	12	21	13	8	5
Citrus medica	Juice	n-H:EA F	14	16	21	12	14	12	11	18	25	14	10	11
Ciprofloxacin (20 µg/ml)			11	10	12	9	14	11	7	10	9	22	8	14
Doxycycline (20 µg/ml)			14	12	9	8	11	5	15	13	4	20	11	19

n-H:EA F – n-hexane:ethylacetate fraction; J:DMSO-juice : Dimethyl sulphoxide ; BC-*Bacillus cereus* ;BS-*Bacillus subtilis* ; EN-*Enterococcus faecalis* ;ML-*Micrococcus luteus*;SA- *Staphylococcus aureus*; SE-*Staphylococcus epidermidis*; EC-*Escherichia coli*; KP-*Klebsiella pneumoniae* ;PS-*Pseudomonas aeruginosa*; SM-*Serratia marcescens* ; SP-*Salmonella paratyphi*; ST-*Salmonella typhi*

Table 5: Antibacterial activity in the crude distilled water extract of selected *Citrus* species

Plant Name	Part used	Extract	Zone of Inhibition(mm)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	crude	30	45	30	40	30	45	29	22	23	29	37	45
		J:DMSO	29	40	29	35	25	45	25	24	27	28	32	44
		n-H:EA F	8	8	12	7	11	7	8	8	18	9	10	5
Citrus medica	Juice	crude	18	17	16	18	18	16	19	18	27	12	12	20
		J:DMSO	15	13	13	15	16	14	15	15	24	10	10	15
		n-H:EA F	23	20	29	22	17	23	23	28	24	25	8	14
Ciprofloxacin (20 µg/ml)			11	10	12	9	14	11	7	10	9	22	8	14
Doxycycline (20 µg/ml)			14	12	9	8	11	5	15	13	4	20	11	19

Table 6: Minimum inhibitory concentration of effective n-Hexane juice extract

Plant Name	Part used	Extract	MIC (mg/ml)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	n-H:EA F	4	4	8	4	4	8	4	>8	>8	4	4	4
Citrus medica	Juice	n-H:EA F	4	4	2	4	4	4	4	8	>8	8	8	4

Table7: Minimum inhibitory concentration of effective ethyl acetate juice extract

Plant Name	Part used	Extract	MIC (mg/ml)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	n-H:EA F	4	4	4	4	8	8	4	-	-	4	4	4
Citrus medica	Juice	n-H:EA F	4	4	4	8	8	2	8	8	>8	4	8	8

n-H:EA F – n-hexane:ethylacetate fraction; J:DMSO-juice : Dimethyl sulphoxide ; BC-*Bacillus cereus* ;BS-*Bacillus subtilis* ; EN-*Enterococcus faecalis* ;ML-*Micrococcus luteus*;SA- *Staphylococcus aureus*; SE-*Staphylococcus epidermidis*; EC-*Escherichia coli*; KP-*Klebsiella pneumoniae* ;PS-*Pseudomonas aeruginosa*; SM-*Serratia marcescens* ; SP-*Salmonella paratyphi*; ST-*Salmonella typhi*

Table 8: Minimum inhibitory concentration of effective distilled water juice extract

Plant Name	Part used	Extract	MIC (mg/ml)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	crude	1	0.5	1	0.125	1	0.125	0.5	-	1	1	2	2
		J:DMSO	1	0.5	1	0.125	1	0.125	0.5	4	1	1	2	2
		n-H:EA F	>8	8	>8	>8	8	4	4	8	>8	>8	-	-
Citrus medica	Juice	crude	1	0.125	1	0.125	1	0.125	1	-	1	1	0.5	0.5
		J:DMSO	1	0.125	1	0.125	1	0.125	1	0.5	1	1	0.5	0.5
		n-H:EA F	4	4	8	8	>8	8	4	8	>8	>8	>8	8

Table 9: Minimum bactericidal concentration of effective n-Hexane juice extract

Plant Name	Part used	Extract	MBC (mg/ml)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	n-H:EA F	S 4	S 4	R 8	R 4	R 4	R 8	S 4	-	-	R 4	R 4	R 4
Citrus medica	Juice	n-H:EA F	S 4	S 4	S 2	R 4	R 4	R 4	S 4	R 8	-	R 8	R 8	R 4

n-H:EA F – n-hexane:ethylacetate fraction; J:DMSO-juice : Dimethyl sulphoxide ; R-Resistant; S-Suceptible ;BC-*Bacillus cereus* ;BS-*Bacillus subtilis* ; EN-*Enterococcus faecalis* ;ML- *Micrococcus luteus*;SA- *Staphylococcus aureus*; SE-*Staphylococcus epidermidis*; EC-*Escherichia coli*; KP-*Klebsiella pneumoniae* ;PS-*Pseudomonas aeruginosa*; SM-*Serratia marcescens* ; SP-*Salmonella paratyphi*; ST-*Salmonella typhi*

Table 10: Minimum bactericidal concentration of effective ethyl acetate juice extract

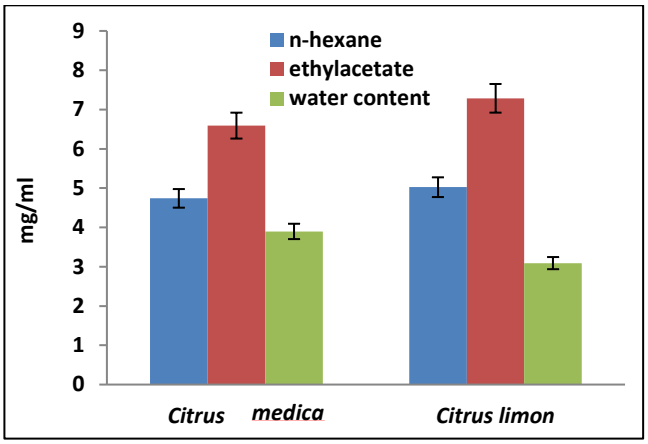
Plant Name	Part used	Extract	MBC (mg/ml)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	n-H:EA F	S 8	R 4	R 4	R 4	R 8	S 8	R 4	-	-	R 4	R 4	R 4
Citrus medica	Juice	n-H:EA F	S 4	S 4	R 4	R 8	R 8	R 2	R 8	R 8	-	R 4	R 8	R 8

Table 11: Minimum bactericidal concentration of effective distilled water juice extract

Plant Name	Part used	Extract	MBC (mg/ml)											
			Gram positive						Gram negative					
			BC	BS	EN	ML	SA	SE	EC	KP	PS	SM	SP	ST
Citrus limon	Juice	crude	S 2	S 4	R 1	S 4	R 1	S 8	S 0.5	-	R 1	R 1	R 2	R 2
		J:DMSO	S 2	S 4	R 1	S 4	R 1	S 8	S 0.5	R 4	R 1	R 1	R 2	R 2
		n-H:EA F	-	R 8	-	-	-	-	S 4	-	-	-	-	-
Citrus medica	Juice	crude	S 2	S 4	R 1	S 4	R 1	S 4	R 1	-	R 1	R 1	R 0.5	R 0.5
		J:DMSO	S 2	S 4	R 1	S 4	R 1	S 4	R 1	R 0.5	R 1	R 1	R 0.5	R 0.5
		n-H:EA F	S 8	S 8	-	-	-	-	S 4	-	-	-	-	-

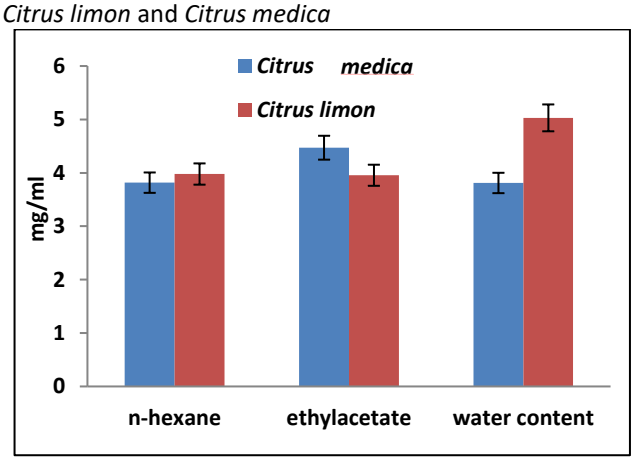
n-H:EA F – n-hexane:ethylacetate fraction; J:DMSO-juice : Dimethyl sulphoxide ; R-Resistant; S- Suceptible; BC-*Bacillus cereus* ;BS-*Bacillus subtilis* ; EN-*Enterococcus faecalis* ;ML- *Micrococcus luteus*; SA- *Staphylococcus aureus*; SE-*Staphylococcus epidermidis*; EC-*Escherichia coli*; KP-*Klebsiella pneumoniae* ;PS-*Pseudomonas aeruginosa*; SM-*Serratia marcescens* ; SP-*Salmonella paratyphi*; ST-*Salmonella typhi*

Figure 4: Presence of total phenols concentration in n-hexane, ethyl acetate and distilled water juice fractions of *Citrus limon* and *Citrus medica*



Total phenol expressed as mg/ml phenol in terms of catechol

Figure 5: Presence of total flavonoids concentration in n-hexane, ethyl acetate and distilled water juice fractions of *Citrus limon* and *Citrus medica*



Flavonoids content expressed as Quercetin equivalent mg/ml

In addition, [27] carried out experiments to test the antibacterial activity of different extract of locally available citrus fruits such as citron (*Citrus medica*), satkora (*Citrus macroptera*) and adajamir (*Citrus assamensis*) against *Bacillus spp*. and *E. coli*. These results were agreed with our results as the juice of *C. limon* and *C.medica* was more effective against selected microorganisms in study. This could be due to the acidic pH of this juice that will affect the charges of the amino acids that constitute the peptidoglycan, and it may affect the active sites of enzymes leading to defect in their activity [28]. The resistance of Gram-negative bacteria to plant extracts was not unexpected. In general, this class of bacteria is more resistant than Gram-positive bacteria. Such resistance could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism [29]. The juice extracts prepared in increasing order of polarity exhibited broad spectrum of antimicrobial activity against all the selected microorganisms. This antimicrobial response of the extracts prepared in both increasing of solvent polarity was found due to presence of phytoconstituent which is almost comparable. Presence of total phenol in n-Hexane, ethyl acetate and distilled water juice fractions of *Citrus medica* and *Citrus limon* (Fig.4) was observed. n-Hexane and distilled water juice fractions of *Citrus limon* showed higher phenolic content i.e. 3.97mg/ml and 5.02mg/ml respectively as compared to *Citrus medica* n-Hexane (3.82mg/ml) and distilled water (3.81mg/ml) juice fractions while *Citrus medica* ethyl acetate juice fractions showed highest phenol concentration i.e. 4.47mg/ml in comparison with *Citrus limon* ethyl acetate juice fractions (3.95mg/ml).Presence of total flavonoid in ethyl acetate, methanol and distilled water juice fractions of *Citrus medica* and *Citrus limon* (Fig.5) was observed. n-Hexane and ethyl acetate juice fractions of *Citrus limon* showed higher flavonoid content i.e. 5.02mg/ml and 7.28mg/ml respectively as compared to *Citrus medica* n-Hexane (4.73mg/ml) and ethyl acetate (6.59mg/ml) juice fractions while *Citrus medica* distilled water juice fractions showed highest flavonoid content i.e. 3.89mg/ml in comparison with *Citrus limon* ethyl acetate juice fractions (3.09mg/ml). However, this difference may be because of the difference in the phytochemical composition in various part of the plant or may be also due to the extraction method used and/or environmental factors or difference in the genotypes of the *Citrus* sps. used. The *Citrus sps.* juice concentration and their extract exhibited higher antibacterial activity as that of the standard antibiotics used in the study. The difference in the antibacterial activity with the same source when extracted with different solvent has proven that not all phytochemicals that are responsible for antibacterial activity are soluble in a single solvent. Hence solvents of different polarity should be employed as discussed in this study (polar: juice, ethylacetate; non-polar: n-Hexane). Solvent fractionation partition is as good option for better solubility of many of the phytochemicals but it is always necessary to know the phytochemicals extracted by each individual solvent and to understand the role of each solvent in the extraction of an individual or class of phytochemicals [30]. Flavonoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide array of microorganisms *in vitro*. Their activity is due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall similar work was recorded[31].

CONCLUSION

The results obtained in present study clearly demonstrated broad spectrum of antimicrobial activity of *Citrus limon* and *Citrus medica*. Thus MIC, MBC assay are capable of verifying that the compound present in juice has antimicrobial activity and that it gives reliable indication of the concentration of drug required to inhibit the growth of microorganisms. Phytochemical analysis are responsible for the identification of bioactive compound which are responsible for antimicrobial activity of plant. Thus, it may be considered as a natural source of antimicrobials for therapeutic purposes.

ACKNOWLEDGEMENT

The authors are thankful to Dr. A.V.R.L.N Acharya, Head of the Department, B.R.D School of Biosciences, Vallabh Vidhyanagar, Gujarat, India and I/C Principal Mr. Harshad Mandola of Shree P.M.Patel Institute of Biosciences, Anand, Gujarat, India for granting the permission to perform the work and providing excellent facilities.

Source of Support: The author(s) received no financial support for the research, authorship, and/or publication of this article

Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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