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PHYSICOCHEMICAL EVALUATION, GC-MS AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF THE FRUIT PEELS OF *CITRUS MACROPTERA* MONTR. OF MEGHALAYA NORTH EAST INDIA

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ABSTRACT

Day to day most of the bacteria became resistant towards antibiotics. The aim of our study is to evaluate the essential oil from the fruit peels of *Citrus macroptera* and to investigate the antibacterial property on antibiotic resistant bacteria. Physicochemical parameters like colour, solubility, refractive index, boiling point, specific gravity, carbon residue, iodine value, acid value, ester value were judged. The composition of the volatile oil was determined by GC-MS analysis. The antibacterial activity of the volatile oil was performed by agar well diffusion technique using antibiotic resistant bacteria. The oil was colourless, denser than water and insoluble in water but soluble in methanol, chloroform and diethyl ether. The p^H was slightly acidic with refractive index and carbon residue were 1.463+0.0025 and 2.59+0.091 %. The major compounds found in the volatile oil were limonene, α-myrcene, terpinene, cyclohexanol, α- terpeneol, azulenemethanol, nootcatone, α-copaene, caryophyllin, nonanal and careen. The oil showed more potency against gram positive bacteria like *Staphylococcus aureus* and *Bacillus subtilis* as compare to gram negative *Salmonella typhi*, *Echerichia coli* and *Shigella dysenteriae*. The volatile oil from the fruit peels of *Citrus macroptera* possesses good antibacterial property.

KEYWORDS: Antibacterial, Essential oils, Physiochemical parameters, GC-MS analysis, *Citrus macroptera*, fruit peel.

1. INTRODUCTION

Essential oils are volatile liquids obtained from different plant parts and widely used as food flavours (Shunying et al.,2005). They are a complex mixture of compounds, mainly monoterpenes, sesquiterpenes, and their oxygenated derivatives like alcohols, aldehydes, esters, ethers, ketones, phenols and oxides. Other volatile compounds include phenyl propenes and specific sulphur- or nitrogen-containing substances (Delamare et al., 2007). Essential (volatile) oils from aromatic and medicinal plants have been known since antiquity to possess biological activity, notably antibacterial, antifungal, and antioxidant properties (Adel et al., 2017). The composition, structure, as well as functional groups of the oils play an important role in determining their antimicrobial activity. It has been demonstrated that the essential oils exercises their antimicrobial activity by causing structural and functional damages to the bacterial cell membrane. It is also indicated that the optimum range of hydrophobicity is involved in the toxicity of the essential oils (Baratta et al., 1998). All parts of the plant contain an essential oil with a characteristic smell (Oh et al.,1967). The essential oils of the plants have been of great interest for their potential uses as alternative remedies for the treatment of many infectious diseases and pharmaceutical alternative medicine and natural therapies (Prabuseenivasan et al., 2006). The development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents(Sibanda et al.,2007). Certain microorganisms have developed resistance against antibiotics and this has initiated antimicrobial investigations with essentials oils or plants against a wide range of both Gram-negative and Gram-positive bacteria including antibiotic resistant species, fungal species and yeast (Nelson 1997).

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace

elements in materials that were previously thought to have disintegrated beyond identification (Masada 1976).

2. MATERIALS AND METHODS

2.1 *Collection, Identification and Authentication of Plant*

The fruits of the plant *Citrus macroptera* were collected locally from East Khasi Hills, Meghalaya. The plant was identified, confirmed and authenticated by Botanical Survey of India, Shillong, Meghalaya.

2.2 *Extraction of Essential Oil*

The fruit peels of *Citrus macroptera* were thoroughly washed with distilled water, cut into small pieces and about 200 gms in four fractions were subjected to hydro-distillation using a clevenger apparatus for about 4 hours. The steamed and vaporized oil were condensed into liquid by a vertical condenser and collected in a measuring cylinder. Being immiscible and lighter than water, the volatile oil is separated out as an upper layer. The oil was collected (Chutia et al., 2009). Finally it was dried over anhydrous sodium sulphate and kept in an airtight container at 4-8^{0}C until further analysis (Isman 2000).

2.3 *Physicochemical Parameters of Essential Oil*

The physicochemical parameters of the essential oil were carried out as per the standard methods to determine the colour, solubility, refractive index, boiling point, iodine test, pH test, specific gravity, carbon residue, acid value, iodine value, ester value and saponification value.

2.3.1 *Colour*

The colour of the essential oil was observed at normal light, at 254 nm and 366 nm using ultra violet chamber. 0.5 ml of the oil was placed in the Petri disk plate in the UV chamber and observed at 254 nm and 366 nm(Shabbir et al.,2009).

2.3.2 *Solubility*

Solubility of the essential oil was determined by taking two liquids i.e. solvent and the oil in the ratio of 3:1.Approximately 1 ml of the essential oil was taken in a test tube containing 3 ml of water and stirred thoroughly; the same was followed using methanol, chloroform, diethyl ether, separately. (Atti-Santos et al.,2005).

2.3.3 *Boiling point*

5ml of the essential oil was placed in a small test tube. A capillary, sealed at one end is placed open-end down into the essential oil. The test tube is firmly attached to a thermometer with a rubber band such that the thermometer bulb should be even with the test tube's bottom, and this entire assembly immersed in an oil bath (half filled 100ml beaker). As the temperature is slowly increased, a rapid evolution of bubbles from the end of the tube begins. Heating was continued for about 5-10 seconds to be sure that all of the air has been expelled from the capillary, and the vapours of the essential oil become equal to the atmospheric pressure. As the temperature decreases, the bubbles slowed down and the essential oil starts rising into the capillary. At the point when the bubble stops, the thermometer was read and recorded. The above process was repeated 2 more times, and the temperature reading in each case was recorded. The above experiment was carried out on orange, lemon and lime essential oil at atmospheric pressure. (Hesham et al.,2016).

2.3.4 *Refractive index*

The refractometer was standardized with distilled water (1.3315 $n^{29}{}_D$). After cleaning and drying with acetone and cotton, a drop of oil was placed between the prisms of the refractometer. The telescope was rotated to bring the border line of total refraction to the junction of crosswire in the telescope. The refractive index was recorded at room temperature- 27^o C(Mahesh et al.,2012).

2.3.5 *Iodine test*

2-3 drops of extracted essential oil was taken in a test tube. Few crystal of iodine was added. Brown colour indicates the presence of limonene or aromatic compounds. (Abdulrahman, M., 2016).

2.3.6 *Carbon residue percentage*

About 1 ml of sample was taken in a silica crucible. Heated strongly at 450^o C till the vapours and smoke disappeared for 3 hours. The sample was cooled down in a desiccators (Khajeh et al.,2005).

Percentage of Carbon residue was calculated by the following formula;

$$\text{Carbon residue (\%)} = W_1/W_2 \times 100$$

Where, W_1 = weight of carbon residue in crucible
W_2 = weight of sample taken

2.3.7 *Specific Gravity*

For the determination of specific gravity of oils, a clean 5 ml specific gravity bottle was weighted (W_0). The bottle was filled to the brim with water and the stopper was inserted. The water on the stopper and bottle were carefully wiped off and reweighed (W_1). Same process was repeated using oil samples instead of water and weighted again (W_2). The specific gravity of all the oil samples was calculated using the following formula. (Shabbir et al., 2009).

Specific gravity of the test sample= W_2-W_0/W_1-W_0
Where,
W_0 = Weight of empty specific gravity bottle
W_1 = Weight of water + specific gravity bottle
W_2 = Weight of test sample + specific gravity bottle.

2.3.8 *P^H Value*

P^H value was directly recorded by using PH meter. The measurements were repeated three times and the average was recorded. (Mary et al.,2013).

2.3.9 *Iodine value*

The iodine value of a substance is the weight of halogens expressed as iodine absorbed by 100 parts by weight of the substance. Place a small quantity of the test substance, accurately weighed, as specified in the monograph, in a dry 300-mL to 500-mL stoppered flask. Add 15 mL of methanol and dissolve it. 0.1 mL of iodine chloride/bromide was dissolved in 19.99mL of glacial acetic acid and then added to the test sample, insert the stopper, shake the flask gently, and kept in the dark for 30 minutes, unless otherwise specified in the monograph. Add 15 mL of 10% potassium iodide and 100 mL of glacial acetic acid, and titrate with sodium thiosulfate (0.5 mol/l), adding starch as indicator. Note the number of mL required. At the same time carry out the operation in exactly the same manner, but without using oil, and note the number of mL of sodium thiosulfate (0.5 mol/l) required. Calculate the iodine value from the following formula (Barkatullah et al.,2012).

Iodine value= {(B S) x N x 12.69}/ Weight of the sample
Where,
B = 0.1 N sodium thiosulfate required (ml) by blank
S = 0.1 N sodium thiosulfate required (ml) by sample
N = Normality of sodium thiosulfate solution.

2.3.10 *Saponification value*
2g of each oil sample was weighted into a clean dried conical flask and 25 ml of alcoholic potassium hydroxide ($K(OH)_4$) was added. A reflux condenser was attached to the flask and heated for an hour with periodic shaking. The appearance of clear solution indicated the completion of saponification. Then 1 ml of 1 % phenolphthalein indicator was added and the hot excess alkali was titrated with 0.5 M hydrochloric acid (HCl) until it reached the end point where it turned colourless. A blank titration was carried out at the same time and under the same condition. The Saponification value was calculated as:

Saponification value = b - a x 8.05/m

Fats (triglycerides) upon alkaline hydrolysis (either with KOH or NaOH) yield glycerol and potassium or sodium salts of fatty acids (soap) (Abdulrahman, et al.,2016).

2.3.11 *Acid Value*
2.5g of oil was taken in a flask. 50 ml of methylated spirit was added to the flask, shake well and titrated against 0.1N KOH solution using phenolphthalein as indicator. Alkali was added till a pink colour was established for a few seconds. The TAN was then calculated using the following formula (Majidi et al.,2015).

Acid Value =V X N X 56.1/W

Whereas,
V = volume of potassium hydroxide used
N = normality of Potassium hydroxide
W = weight in g of the sample

2.3.12 *Ester value*
1.5 grams of essential oil was weighed, and was introduced into a glass flask. It was added through burette 25ml of ethanol solution of KOH (0.5mol/l). The condenser was adapted and was placed the ball on the heating mantle and allowed to heat for one hour. After cooled, 20ml of distilled water and 5 drops of 0.2% PP. were added to it. Finally, as the excess of KOH solution with hydrochloric acid 0.5mol/l. alongside the operation cited, blank was made under the same conditions and with the same reagents (Kumar et al., 2014).

2.4 *Gas Chromatography-Mass Spectrometry Analysis (GC/MS)*
GC/MS analyses were performed on a Thermo Fischer capillary gas chromatograph directly coupled to the mass spectrometer system (model GC ULTRA S/N 20062969; PolarisQ S/N 210729). HP-5MS non polar fused silica capillary column (50 m x 0.32 mm, 1.25 µm film thickness) was used under the following conditions: oven temperature program from 40°C (2 min) to 280°C at 5°C/min, and the final temperature kept for 10 min; injector temperature 250°C; carrier gas He, flow rate 1mL/min; the volume of injected sample was 1.5µl of diluted oil in hexane; split less injection technique; ionization energy 70eV, in the electronic ionization (EI) mode; ion source temperature 200°C; scan mass range of m/z 40-650 and interface line temperature 300°C. The constituents of essential oils were identified based on their Kovats Index, calculated in relation to the retention time of a series of alkenes (C4- C28) as reference products, in comparison with those of the chemical compounds gathered by Adams table, and the similarity of their mass spectra with those gathered in the NIST-MS library, or reported in the literature (Derwich et al.,2010).

2.5 *Anti-Bacterial Screening*
The antibacterial activity was performed by employing Agar well diffusion method.

2.5.1 *Test organisms*
The following Gram positive and Gram negative bacteria were used for antibacterial screening.

Staphylococcus aureus (S1) NCTC-6571, *Bacilus subtilis* (B1) NCTC-10341, *Escherichia Coli* (E.coli2) NCTC-832. *Salmonella typhi* (SL2) NCTC-59, *Shigella dysenteriae* (SG-2) NCTC-7.

2.5.2 *Standard drug solution*
Streptomycin in a concentration 2mg/ml was used as a standard drug.

2.5.3 *Preparation of inoculums*
The peptone water medium was sterilized by autoclaving at 15p.s.i. pressure and at 121 ^{0}C for 15 minutes. A loop full of organism was transferred from a laboratory maintained mother culture into different Bijou bottles

containing sterilized peptone water medium. The Bijou bottles were incubated for 37 ^{0}C for 48hours.

2.5.4 *Bacteriological testing by Agar well diffusion method*

Agar well diffusion method was elucidated with some modifications. The Nutrient agar plates were prepared by pouring 15 ml of molten media into sterile Petri-plates. After solidification 20µL of microbial broth containing 108-109 cfu/ml was spread on the surface of nutrient media. The microorganisms were fixed on the surface of solid media by incubating at 37^{0}C. Wells or cups of 5mm size were made with sterile borer into agar plates containing the bacterial inoculums; 30µL volume of the essential oil of density (0.87g/ml) measured by weight: volume ratio was mixed with DMSO and poured into a well. Streptomycin (2mg/ml) was used as a positive control and DMSO (3%) was used as a negative control. The plates thus prepared were kept at room temperature for ten minutes allowing the diffusion of the oil into the agar media. After incubation of 24 hrs at 37^{0}C, the plates were observed. The different zones of inhibition were measured(Suganya et al.,2012).

3. RESULTS

The fruit peels of *Citrus macroptera* were subjected to extraction by using Clevenger apparatus to perform hydro-distillation and subjected to preliminary physicochemical analysis. The percentage yield of the oil was found to be 1.575 % (w/v). In this study, we have analysed the various physicochemical parameters such as colour, solubility, pH, iodine value, refractive index, boiling point, specific gravity, carbon residue, saponification value, acid value and peroxide value. The values were recorded in Table 1.

Table 1: Physicochemical evaluation of *Citrus macroptera* essential oil.

S. No	Physicochemical parameters	Results
01	% yield	1.575 %
02	Colour of extracted oil	Colourless at visible light, 254 nm and 366 nm.
03	Solubility	Insoluble in water and chloroform, soluble in diethyl ether and methanol
04	pH of extracted oil at 25^{O}C	6.78 ± 0.096
05	Iodine test	Brown colour indicates the presence of limonene or aromatic compound
06	Specific gravity at 27^{o}C	1.46 ± 0.0098
07	Boiling point (^{O}C)	175.34 ± 9.56
08	Refractive index at 27^{o}C	1.46 ± 0.0025
09	Carbon residue (%)	2.59 ± 0.091
10	Saponification value	165.75 ± 5.962
11	Acid value	1.99 ± 0.522
12	Ester value	164.05 ± 11.58
13	Iodine value	106.27 ± 4.876

3.1 *Gas Chromatography-Mass Spectrometry (GC-MS)*

Gas chromatography–mass spectrometry (GC-MS) is used to perform a specific test which identifies the actual presence of a particular substance in a given sample. The retention data, area of abundance (%), molecular mass and chemical composition of *Citrus macroptera* essential oil were presented in Table 2. The GC/MS analysis of the essential oil revealed that the oil is composed of limonene (22.69%), α-terpineol (10.27%), α- myrcene (7.69%), terpinene (4.48%), cyclohexanol (3.88%), carene (1.48%) and β-caryophyllene (1.22%) as the main components Figure-1.

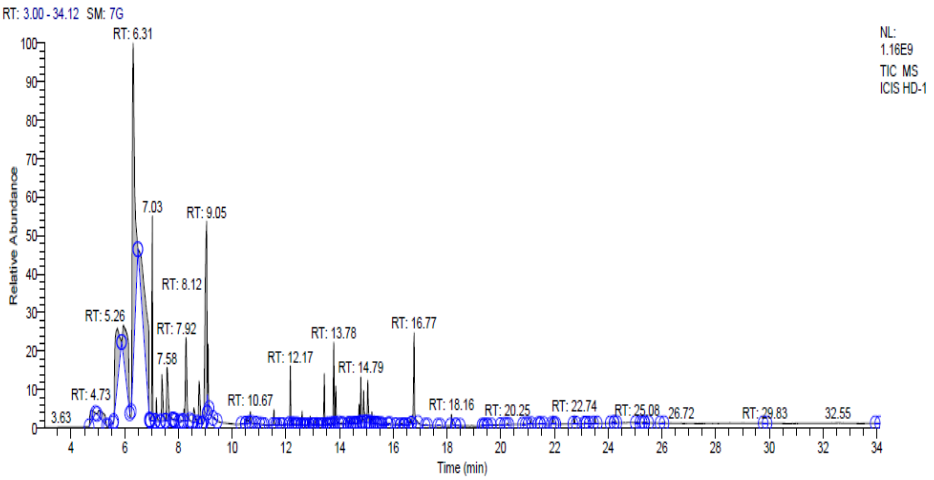

Fig 1: GC-MS of *Citrus macroptera* essential oil.

Qual Peak Table

RT	Peak Area	Area %	Peak Height
4.73	308595798.29	1.14	37747617.07
5.26	400465829.49	1.48	26067962.11
5.44	43130564.85	0.16	7179825.21
5.65	2077407051.43	7.69	206532691.47
6.13	2060559315.24	7.62	179668861.09
6.31	6133448152.76	22.69	979776293.55
6.89	3559836475.43	13.17	242014966.63
6.94	8034249.24	0.03	7946175.74
7.03	1209666729.10	4.48	616010822.50
7.17	173819653.55	0.64	70197288.60
7.39	479599308.93	1.77	141863530.25
7.58	735165084.37	2.72	160110031.62
7.81	4991561.32	0.02	2866384.87
7.92	25971849.38	0.10	6150620.46
8.12	10490560.05	0.04	3873653.59
8.28	1049497417.84	3.88	249264618.28
8.57	182956251.20	0.68	41895167.47
8.77	500649241.38	1.85	126373737.50
8.88	18591512.96	0.07	17461381.23
9.05	2776712964.49	10.27	581902067.43
9.10	379281515.07	1.40	200789199.42
9.34	23210921.98	0.09	4820588.62
10.36	7237392.83	0.03	1127616.33
10.55	73661153.12	0.27	18393586.24
10.67	66405984.11	0.25	35190319.40
11.00	7188638.85	0.03	2157051.20
11.17	6640832.06	0.02	3296361.86
11.55	87062365.73	0.32	46517129.03

RT	Peak Area	Area %	Peak Height
11.75	47707725.83	0.18	22759459.79
11.95	20592054.46	0.08	2832102.03
12.17	330883825.21	1.22	176555329.26
12.27	17245735.29	0.06	8127692.25
12.36	4108284.71	0.02	2421263.53
12.42	4278968.53	0.02	1927002.38
12.54	16541124.22	0.06	5059522.84
12.60	78502610.55	0.29	42382066.22
12.70	4122253.33	0.02	1849545.78
12.82	55989016.58	0.21	19252464.02
12.91	66274526.96	0.25	26887730.28
13.03	10774379.78	0.04	4282181.94
13.14	82342142.72	0.30	23184031.61
13.23	28408856.77	0.11	12659140.44
13.36	38760228.64	0.14	16713916.36
13.43	328892158.43	1.22	154319913.53
13.56	17219301.87	0.06	9212447.74
13.61	8263224.22	0.03	3761526.86
13.70	9532384.14	0.04	5810140.21
13.78	505129103.50	1.87	246473786.22
13.86	271106056.44	1.00	113107276.87
14.09	10541834.23	0.04	6162866.50
14.25	62674144.56	0.23	19828708.45
14.36	34899811.35	0.13	16427967.27
14.47	44655526.88	0.17	11081524.71
14.54	18244645.95	0.07	9529845.88
14.59	8323364.10	0.03	4636680.88
14.73	141614223.48	0.52	57819244.58
14.79	290611416.34	1.08	138824199.84
14.90	255604204.56	0.95	96691594.63
15.05	338550829.77	1.25	127379178.68
15.14	42794317.92	0.16	21085170.53
15.20	76943565.50	0.28	34372215.87
15.33	5988914.44	0.02	2427432.62
15.40	7534639.77	0.03	3438287.29
15.45	4777788.61	0.02	2141063.75
15.54	5812896.22	0.02	2931642.02
15.71	46358172.46	0.17	5249410.03
15.84	11292982.62	0.04	3239998.28
16.13	15184120.23	0.06	4361335.03
16.30	24542493.58	0.09	6854621.14
16.40	5838350.90	0.02	2845205.71
16.45	12051074.81	0.04	3052459.94
16.63	44340750.91	0.16	14221135.42
16.77	754574905.81	2.79	272587467.47

3.2 *In vitro* Antibacterial activity

This study showed that the oil from fruit peels of *Citrus macroptera* was more potent against gram positive bacteria such as *S. aureus and B. subtilus* and less active for gram negative *S. typhi*, *E.coli* and *S. dysentria* Table-2, Figure-2 and Figure-3.

Table 2: *In vitro* Antibacterial Sensitivity test of *Citrus macroptera* fruit peels Essential oil in millimetres.

Micro-organism strains	Test (Essential Oil)	Control (DMSO-3%)	Standard (streptomycin 100µg/ml)
Staphylococcus aureus (S1)-(+ve)	10	0	20
Bacillus subtilus (B1)-(+ve)	12	0	21
Salmonella typhi (SL2)-(-ve)	5	0	27
Echerichia coli (E.coli2)-(-ve)	7	0	25
Shigella dysentria (SG2)-(-ve)	8	0	19

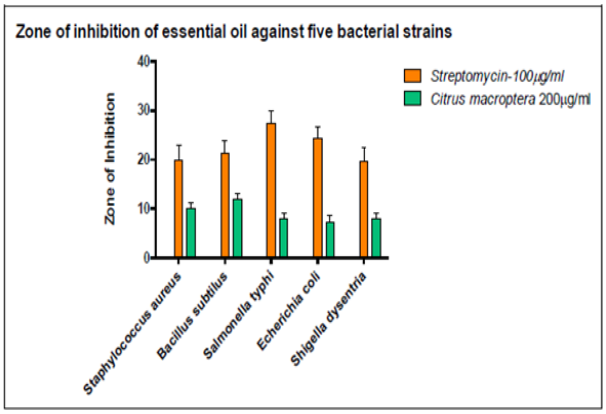

Fig 2: Zone of Inhibition of essential oil against bacterial strains.

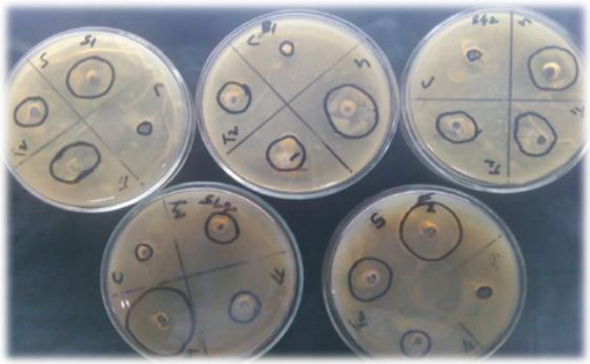

Fig 3: Image for inhibition zones (antibacterial activity).

4. DISCUSSION

In the present study, the percentage yield of the essential oil from fruit peels of *Citrus macroptera* by hydro distillation was found to be 1.575%. But this may be due to geographical distribution, fruit collection time, quantity of plant used, duration of extraction, climatic variation, temperature, humidity, etc. It was found that the essential oil was colourless at normal light. But at 254nm and at 366nm we found variation in colours from pale yellow to light yellowish. This is due to absorption of different wave length by the chromophore group present in terpends(Bones et al., 2010). It is insoluble in water, chloroform, soluble in diethyl ether and methanol. Brown colour confirms the presence of limonene. The pH was slightly acidic. The specific gravity was higher than water(Osagie et al., 1986). Carbon residue determines the presence of nonvolatile impurities which was found very less (2.59 % w/w). The oil is composed of a number of unsaturated compounds and it was confirmed from its iodine value(Adebayo et al., 2011; Akinyeye et al., 2011).

The GC-MS of fruit peels of *Citrus macroptera*essential oil revealed that there are 42 number of compounds (table-3) and major compound were limonene (22.69%), α-terpineol (10.27%), α- myrcene (7.69%), terpinene (4.48%), cyclohexanol (3.88%), etc. as compared to previous studies(Virendra S. Rana, *et al.,2012*) they found 47 compounds from fresh fruit peels along with *Citrus maxima* and major components were limonene (55.3%), oxygenated monoterpenes (15.6%), geranial (3.5%), etc. [F](Jean Waikedre, *et al.,* 2010) also stated that essential oil obtained from leaves of *Citrus macroptera*contains 35 compounds representing 99.1% of the essential oil, the oil was rich in monoterpenes (96.1%), with β-pinene as major component (33.3%), and poor in limonene (2.4%.) but in case of our study limonene is major compound (22.69%).

From the antibacterial activity carried out by agar well diffusion method it was recorded that the oil from fruit peels of *Citrus macroptera* was active against gram positive bacteria (*S. aureus and B. subtilus*) and lowest for *S. typhi* (gram negative bacteria). In case of essential oil obtained from leaves of *Citrus macroptera* showed no activity against selected bacteria (Jean Waikedre, *et al.*, 2010). This may be due to presence of various types of isoprenoid compounds.

5. CONCLUSION

In conclusion, our study is the first report on the physiochemical parameters, chemical composition and *in vitro* antibacterial activity of the essential oil from fruit peels of *Citrus macroptera* in one research paper. The physiological parameters helped in formulating the pharmacopoeial standard for the drug. The essential oil was characterized by the high content of limonene, α-terpineol, α- myrcene, terpinene, cyclohexanol, carene and β-caryophyllene. This study showed that essential oil displayed active inhibitory activity against gram positive

bacteria but with lesser inhibitory action with gram negative bacterial strain. So, it has been clearly seen that the essential oil of *Citrus macroptera* possesses active antibacterial activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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