International Journal of Botany Studies
ISSN: 2455-541X, Impact Factor: RJIF 5.12
www.botanyjournals.com
Volume 1; Issue 7; November 2016; Page No. 35-41

Evaluation of antioxidant and antimicrobial properties in *Cymbopogon citratus* (DC.) Stapf

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Abstract
Cymbopogon citrates (DC.) Stapf is an important and oldest species of Cymbopogon grass belonging to the family of Poaceae and commonly called western Indian lemongrass or lemongrass. The quality of lemongrass oil is determined by the content of Citral, an isomer of geranial (40-62%) and neral aldehyde (25-38%) responsible for the lemon odor. Other major components include in the essential oil include β-myrcene and Geraniol. The oil of *C. citratus* is known to posess varied biological activities. During the present investigation, the essential oil of *C. citratus* oil were fractionated into terpene-less and terpene-containing parts and investigated for antioxidant and antimicrobial activities. The *in vitro* antioxidant and ROS radical scavenging activity of the essential oil were evaluated by DPPH assay. The antimicrobial property of the pathogenic strains like *Enterococcus faecalis*, *Staphylococcus aureus, Shigella dysenteriae*, *Salmonella typhii*, *Trichophyton rubrum* and *Cryptococcus neoformans* were tested against the essential oil. The present investigation highlights the significance of *C. citratus* essential oil and their fractions as antimicrobial and antioxidant agent.

Keywords: *Cymbopogon citratus***,** Essential oil, antioxidant, antimicrobial agent

1. Introduction
Wild Cymbopogon species are characterized as highly polymorphic taxa because of their variable essential oil composition owing to different environmental and genetical factors (Pluhár *et al.*, 2005, 2007) [24, 25]. In India, native Cymbopogon species have been described from various habitat conditions (Pluhár *et al.*, 2005, 2007) [24, 25]. *Cymbopogon citratus* (DC.) Stapf is commonly called as lemongrass and considered as most highly specialized tribe under grasses (Bews, 1929; Hartley, 1950) [5, 17]. The grass belongs to well-defined monophyletic tribe exhibiting greater morphological and adaptive variations due to hybridization, polyploidy etc. Wide scale GC screening has been carried out to study the active components present in the essential oil (Vinutha *et al.*, 2013) [29]. According to the recent review, a total of 158 compounds have been identified in *C. citratus* oil by several workers (Rajeswara, 2013) [27]. The essential oil of *C. citratus* contains Citral, β-Myrcene (characteristic and active ingredient of lemongrass oil), Geraniol, Geranyl acetate, Piperitone, Limonene, Elemicin, Monoterpene and Sesquiterpenes alcohols as major constituents (Tajidin *et al.*, 2012; Quintanilla *et al.*, 2012 and Vyshali *et al.*, 2013) [31, 26, 30].
Traditional uses from centuries old herbal medicines in old world countries gave clues to their potential for the new world remedies. The lemongrass oil is gaining increasing interest because of their potential for multipurpose functional use, such as for its biological activities and as flavor and fragrance ingredients. These activities are attributed to the presence of essential oil chemical compounds, their structural and functional groups. Many studies have reported the antimicrobial activity of lemongrass oil originating from different parts of the globe against diverse range of microorganisms comprising Gram positive and Gram negative bacteria, yeast and fungi (Bassole *et al.*, 2011; Falcão *et al.*, 2012) [3, 14]. The oil showed fungicidal and anti-aflatoxigenic effects against *Aspergillus flavus* (Paranagama *et al.*, 2003). The major components of oil with α-Citral and β-Citral have shown to possess antibacterial activity (Onawunmi *et al.*, 1984). The α-Citral (geranial) and β-Citral (neral) components individually elicit antibacterial action.
The antioxidant and radical-scavenging properties are of great interest to health and food science researchers. Studies have revealed that the essential oil of *C. citratus* and their components are known for their antioxidant activities (Mahmoud *et al.*, 2010; Koh *et al.*, 2012) [21, 19]. *C. citratus* has known to possess cytoprotective and anti-inflammatory property by reducing the oxidative stress (Tiwari *et al.*, 2010) [32]. The essential oil of lemongrass protects DNA against chemically-induced damage and also exhibits anticarcinogenic activity (Bidinotto *et al.*, 2011) [6] and beneficial in reducing the blood cholesterol level (Costa *et al.*, 2011) [11]. During the present investigation the essential oil of *C. citratus* and its fractions were analyzed for antimicrobial and antioxidant activity.

2. Materials and Methods
2.1 Collection and maintenance of Germplasm
The *Cymbopogon citratus* collected from Moodabidri (D.K.) and were maintained in the Departmental garden of Microbiology and Biotechnology, Bangalore University, Bangalore under uniform conditions for further studies. The plant sample was taxonomically identified and deposited at the National Ayurveda Dietetics Research Institute, Bangalore [Ref RRCBI-Mus/06].

2.1.1 Essential oil extraction and analysis
Essential oil extraction: The shade-dried leaves of lemongrass were chopped into small (10cm long) pieces, weighed and was subjected to hydro-distillation for 3 hrs using Clevenger's apparatus (Clevenger, 1928) [10]. The

obtained oil was collected and dried over anhydrous Sodium sulphate and kept at 4°C until analysis. The essential oil yield was calculated on the basis of dry weight of the material (V/W) using the formula,

Essential oil content = a×(V/W) (100/b)

Where,
a = volume of the oil (mL) collected
b = weight of the sample (g) taken

2.1.2 Essential oil analysis by Gas Chromatography (GC-FID)

GC analysis of the oil samples was performed on an Agilent Technologies gas chromatograph Model 6890N equipped with dual FID. A CP Sil8CB column (30m×0.25mm×0.25µm film thickness) coated with dimethylpolysiloxane with 5% diphenyl as the stationary phase. Helium was used as the carrier gas at a flow rate of 1 mL per min. (constant flow). Temperature programming was done from 50°C (2 min) to 280°C at 10°C/min. Injector and detector temperature were maintained at 250°C and 280°C respectively. Samples of 1 µL dissolved in hexane were injected using a split ratio of 10:1.

2.1.3 Identification of compounds

Component identification was done by comparison of linear retention indices of GC peaks with those of standard compounds and literature (Fig 1) (Adams, 2007).

2.1.4 Fractionation of essential oil

The essential oil of *C. citratus* was separated into two major fractions (non-polar and polar) using silica column chromatography and subjected to TLC, GC and GC-MS analysis. Using TLC, oxygenated terpenes and non-oxygenated terpenes of the essential oil were separated and identified by comparing Rf value with the literature.

2.2 Antioxidant activity

The *in vitro* antioxidant and ROS radical scavenging activities of *C. citratus* essential oil and its fractions (terpene-less and terpene-containing) were evaluated by DPPH assay using Ascorbic acid as Standard and positive control. The tests were performed in triplicates and averaged. Using DPPH assay the ability of essential oil to scavenge synthetic free radical 1,1-diphenyl 2-picryldihydrazyl (DPPH) was determined using standard method (Hsu *et al.*, 2007). The essential oil samples and the Standard was tested at various concentrations (10-50µg/mL) and adjusted to 100µl using methanol. To this 0.1mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C in dark. The absorbance was measured at 517nm against blank. Radical scavenging activity was expressed in percentage (%).

Percentage of inhibition (%) =
[(Control–Sample)/Control] × 100

Where,
Control is the absorbance of DPPH radical + methanol; Sample is the absorbance of DPPH radical + sample/standard.

Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting percentage of inhibition against sample concentration.

2.3 Antimicrobial activity

The antimicrobial activity of *C. citratus* essential oil and its fractions (terpene-less and terpene-containing) were evaluated against two strains of Gram-positive bacteria, *Enterococcus faecalis* (clinical isolate) and *Staphylococcus aureus* (MTCC 3160) and two strains of Gram-negative bacteria *Shigella dysenteriae* (clinical isolate) and *Salmonella typhii* (clinical isolate), and a fungus *Trichophyton rubrum* (MTCC 3018) and an yeast strain *Cryptococcus neoformans* (clinical isolate). MTCC cultures were procured from microbial type culture collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, Punjab.

For inoculum preparation, the stock cultures of bacterial and fungal strains were maintained at 4°C on slopes of Nutrient Agar (NA) and Sabouraud Dextrose Agar (SDA) (Himedia) respectively. Active cultures for experiments were prepared by transfering the cells from stock cultures to Mueller-Hinton broth for Bacteria and Sabouraud dextrose broth for Fungi and incubated without agitation for 24 h at 37°C and for 48 h at 25°C respectively. Active cultures of bacterial and fungal strains were adjusted to 105 CFU/mL.

The Antimicrobial activity was performed by Agar disc diffusion (Bauer *et al.*, 1966) and Agar dilution method according to the guideline of NCCLS (NCCLS, 2004).

2.3.1 Disc Diffusion Method

Test plates were prepared with NA (for Bacteria) and SDA (for fungus) and inoculated (100 µL) with a bacterial/spore suspension in sterile dissolution of 0.9% saline. The concentration of suspension was adjusted to 105CFU/mL. 6 mm diameter sterile filter paper discs (Whatman No. 1 filter paper) were soaked in 10 µL of essential oil samples and methanol and air dried and the discs were placed on surface of plates and incubated at 28°C for 48 h. The zone of inhibition was determined against standard drug (Sigma) Fluconazole (30mg/mL) for antifungal activity and Ciprofloxacin (15 µg/mL) for antibacterial activity as positive control and methanol as negative control. The zone of inhibition of microbial growth around the well was measured in mm. The experiment was performed in triplicates to minimize the error rate.

2.3.2 Agar Dilution Method

The agar dilution method was followed to determine the Minimal inhibitory concentration (MIC) of the essential oil. A concentration of 0.5% (v/v) Tween-20 (Sigma) was incorporated into the agar after autoclaving to enhance solubility of the essential oil. A series of dilution of oil ranging from 2% (v/v) to 0.031% (v/v) were prepared in MHA and SDA with 0.5% (v/v) Tween-20. The plates were dried at 35°C for 30 min prior to inoculation with 1-2 mL spots containing approximately 105 CFU of each organism. SDA with 0.5% (v/v) Tween-20 without oil was used as positive control. The inoculated plates were incubated at 35°C for 48h. The MIC was determined as the lowest concentration of oil inhibiting the visible growth of each

organism on the agar plate. The presence of one or two colonies was disregarded.

3. Results and Discussion

3.1 Essential oil extraction

Hydro-distillation of the shade dried leaves of *C. citratus* collected from Moodabidri produced essential oil with strong fresh grassy citrus odor. The plant showed oil content of 1% and Citral content of 0.9±0.02**.** The GC analysis of the essential oil performed showed the presence of oxygenated terpenes (Rf 0.60-0.75) and non-oxygenated terpenes (Rf 0.82). The polar fraction of the oil showed the presence of oxygenated terpenes like Citral (Neral+Geranial), Geraniol, linalool, Iso Citral as the constituents and designated as 'terpene-less fraction' and the analysis of non-polar fraction rich in β-Myrcene (≥94.87%) was designated as 'terpene-containing fraction' (Table 1).

3.2 Antioxidant activity

Antioxidant activity of essential oils is a biological property of great interest as they act as human health protecting factor and reduce the risk of chronic diseases. In the present study, antioxidant capacity of *C. citratus* essential oil and its fractions were evaluated according to the DPPH radical scavenging capacity. The results of antioxidant activity of the tested essential oil samples were expressed as percentage of inhibition (%) and IC_{50} (Inhibition Co-efficient) values, as shown in Table 2.

The results showed that no statistical significant differences exist between the DPPH free radical scavenging activities of the essential oil samples and Std. Ascorbic acid. The *C. citratus* essential oil was able to reduce the stable free radical of 2,2′ - diphenyl -1- picrylhydrazyl to diphenylpicrylhydrazine with an IC50 of 22.57±0.00 µg/mL, a value similar to that of Std. Ascorbic acid (19.07±0.02 µg/mL). The terpene-less fraction of the essential oil also exhibited similar free radical scavenging activity to that of the whole essential oil with an IC_{50} value of 24.9±0.01 µg/mL. The terpene-containing fraction was also found to be efficient in radical scavenging activity with an IC_{50} value of 27.02±0.06 µg/mL (Fig 2 and 3). The result can be attributed to the high antioxidant activity manifested by the monoterpenoid compounds present in essential oil such as Neral, Geranial and Myrcene through synergic action (Ruberto and Baratta, 2000; Koroch *et al.*, 2007) [28, 20]. Thus, the results clearly indicate that *C. citratus* essential oil and its fractions are effective in scavenging free radical and has the potential to act as powerful antioxidant agent.

3.3 Antimicrobial activity

During the investigation, the essential oil and their fractions tested against the pathogenic microorganisms showed positive results. Comparative studies of *C. citratus* essential oil and its terpene-less fraction with the positive control showed good activity against the tested microorganisms while terpene-containing fraction of the oil showed less or no activity. This can be explained due to the action of oxygenated terpenes present in *C. citratus* essential oil and its terpene-less fraction showing overwhelming antimicrobial activity than their hydrocarbon counterparts as proposed earlier (Dorman and Deans, 2000; Hammer *et al.*, 2003) [13, 16].

The *C. citratus* essential oil and its terpene-less fraction showed antibacterial activity against gram negative *S. typhii* and *S. dysenteriae* which generally are less susceptible to antibiotics than gram-positive bacteria by having selectively permeable outer membrane of lipoprotein and lipopolysaccharide and thus regulate access of antimicrobials into the underlying cell structures (Cabeen and Jacobs-Wagner, 2005; Chopra *et al.*, 2001) [8, 9]. This characteristic antimicrobial activity of the terpene-less fraction of the oil is due to the presence of bioactive compounds α-Citral (Geranial) and β-Citral (Neral) in higher percentages. However, the terpene-containing fraction of the oil containing β-Myrcene possesses no antibacterial activity. β-Myrcene individually showing no activity and enhancing the activity when combined with other components has been reported (Grace *et al.*, 1984) [5].

In general, there seemed to be overall agreement between size of inhibition zones obtained using Disc diffusion method and their MIC values. The larger zones of inhibition (Table 3, Fig 4) correlated with lower MIC values (Table 4, Fig 5). One of the possibilities for antimicrobial action of essential oil is the generation of irreversible damage to the membrane of bacterial cells inducing material loss (cytoplasmic), leakage of ions, loss of energy substrate (glucose, ATP) and leading directly to lysis of bacteria (cytolysis). Another possibility of action is inhibition of production of amylase and protease which stop the toxin production, electron flow resulting in coagulation of the cell content (Burt, 2004; Di Pasqua *et al.*, 2007; Hammer *et al.*, 2003; Bakkali *et al.*, 2008) [7, 12, 16, 2]. The antifungal activities are identical to that of bacteria. However, additional phenomena inhibiting the action of yeast strain (*C. neoformans*) is worth mentioning and need further investigation.

Table 1: Chemical composition of *C. citratus* essential oil

RI	Compound	Percentage
990	β-Myrcene	8.8
1259	Neral	30.4
1260	Geraniol	2.2
1385	Geranyl acetate	0.4
1280	Geranial	41.8
1184	(E)-IsoCitral	1.4
1151	Citronellal	0.3
1143	*exo*-IsoCitral	1.0
1165	(Z)-IsoCitral	0.9
1229	Citronellol+Nerol	0.5
	Oxygenated monoterpenes	84.0

Table 2: DPPH scavenging activity of *C. citratus* essential oil

S. No.	Sample	Concentrations (µg/mL)	Inhibition (%)	IC50 value (µg/mL)*
1.	Std. Ascorbic acid	10	14.945	19.07±0.07
		20	57.126	
		30	95.627	
		40	96.572	
		50	97.653	
2.	Total essential oil	10	9.845	22.57±0.08
		20	43.482	
		30	89.683	
		40	94.141	
		50	94.789	
3.	Terpene-less fraction	10	12.884	24.9±0.07
		20	39.126	
		30	91.101	
		40	94.242	
		50	93.533	
4.	Terpene-containing fraction	10	6.907	27.02±0.06
		20	31.223	
		30	90.696	
		40	92.824	
		50	94.445	

*Data expressed as mean±SD. Values not significantly different at P<0.05, n=3 as measured by Tukey's HSD test. Positive control-Ascorbic acid

Table 3: Antimicrobial activity of *C. citratus* essential oil against pathogenic strain

S. No.	Micro organism	Zone of inhibition (mm)				
		Total essential oil	Terpene- less fraction	Terpene-containing fraction	Positive control	Negative control
1.	*Trichophyton rubrum*	11.0±0.88a	9.0±0.62a	-	16±0.95a^	-
2.	*Cryptococcus neoformans*	15.0±0.60b	12.0±0.70b	-	22±1.02b^	-
3.	*Staphylococcus aureus*	10.5±0.52ac	7.5.0±0.54c	4.0±0.75a	12±0.80c*	-
4.	*Enterococcus faecalis*	8.0±0.65c	6.0±0.42dc	3.0±0.63a	14±0.78c*	-
5.	*Salmonella typhii*	11.4±0.80a	7.0±0.86c	-	16±1.00a*	-
6.	*Shigella dysenteriae*	14.0±0.93b	10.0±0.90b	7.0±0.82b	26±1.05d*	-

*Ciprofloxacin, ^Fluconazole (30mg/mL); Negative control (DMSO solvent)
Data represented as mean±SD. Data analyzed by Tukey's HSD. Mean followed by different superscript statistically vary (p<0.05, n=5)

Table 4: Minimal inhibitory concentration (MIC) of *C. citratus* essential oil against pathogenic strains

S. No.	Microorganism	MIC percentage (v/v)	
		Total essential oil	Terpene-less fraction
1.	*Trichophyton rubrum*	0.06±1.4a	0.08±1.02a
2.	*Cryptococcus neoformans*	0.10±1.2b	0.12±1.01b
3.	*Staphylococcus aureus*	0.12±1.3b	0.20±1.05c
4.	*Enterococcus faecalis*	0.20±1.5c	0.25±1.06d
5.	*Salmonella typhii*	0.25±1.2c	0.25±1.05d
6.	*Shigella dysenteriae*	0.50±1.4d	0.25±1.04d

Data represented as mean±SD. Data analyzed by Tukey's HSD. Mean followed by different superscript statistically vary (p<0.05, n=5)

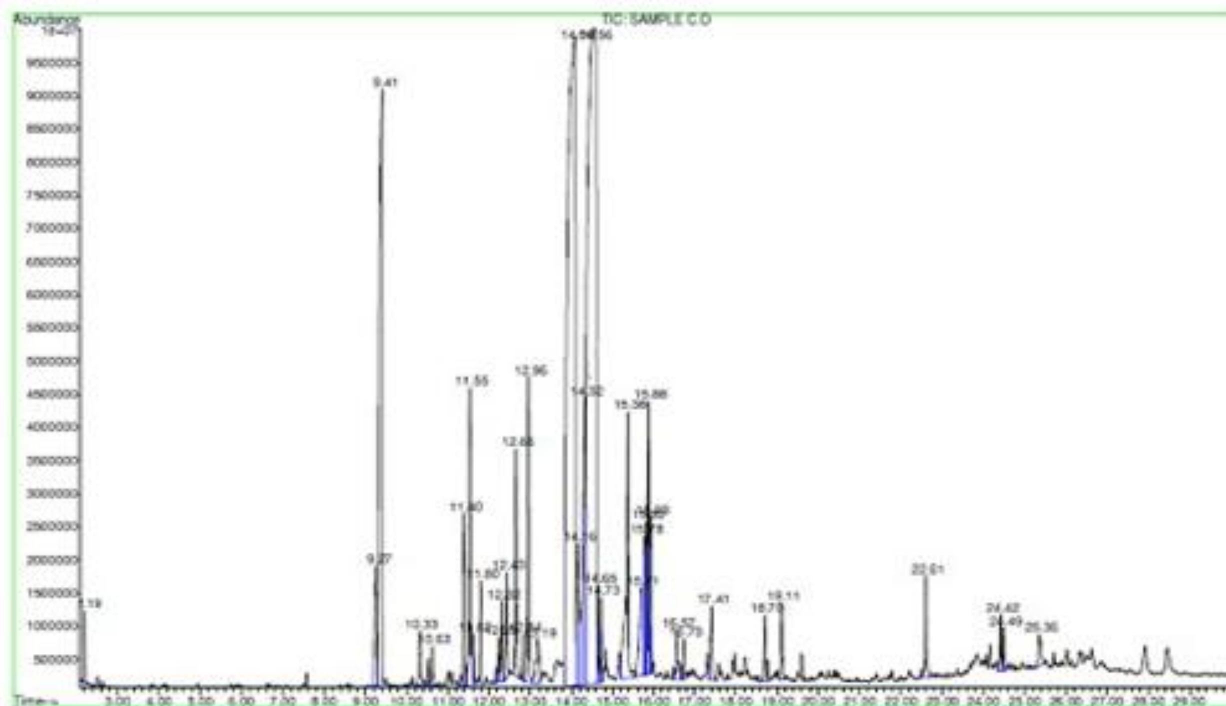


Fig 1: Gas Chromatogram of essential oil from *C. citratus*

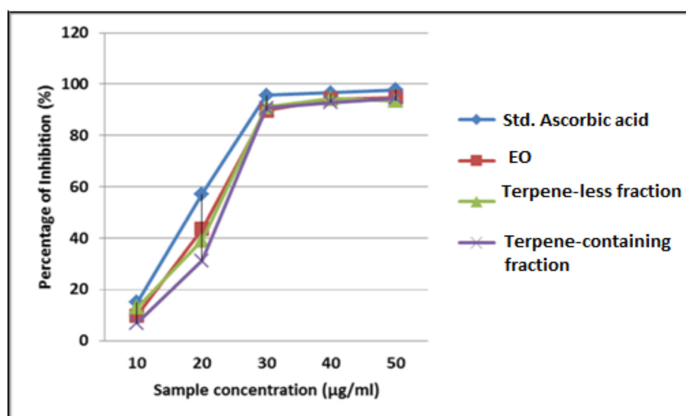


Fig 2: Antioxidant activity of *C. citratus* essential oil and its fractions with in DPPH assay

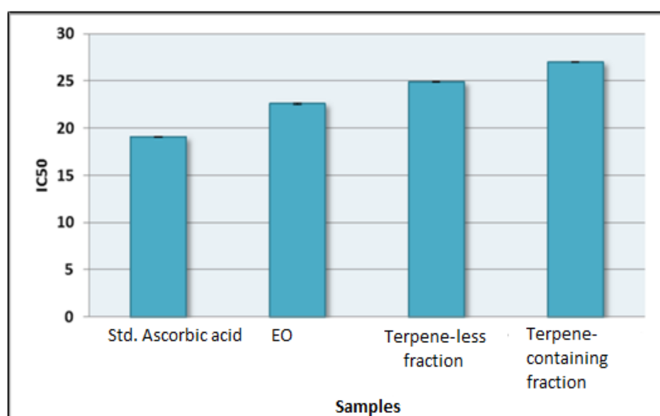


Fig 3: IC_{50} values of DPPH antioxidant activity of *C. citratus* essential oil

Data analyzed by Tukey's HSD test at P<0.05, n=3, Positive control-Ascorbic acid

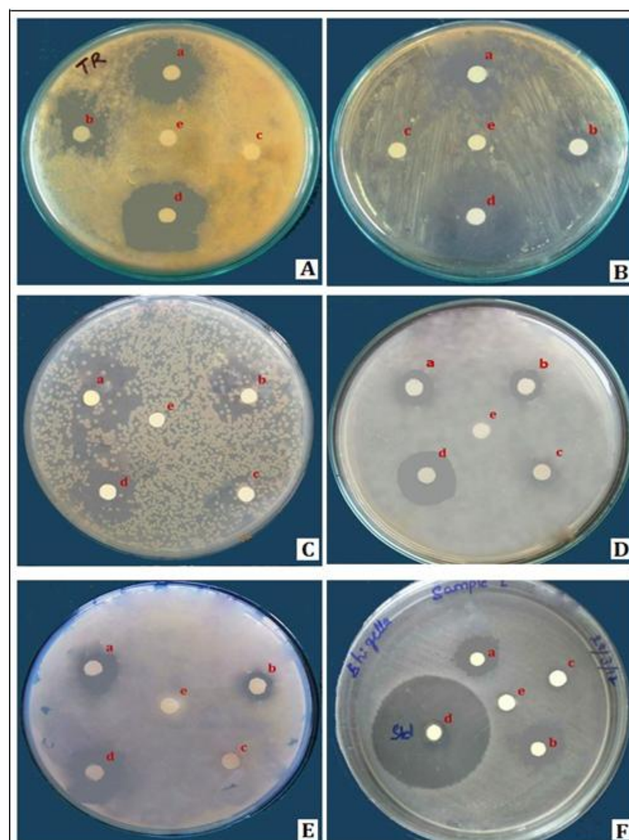
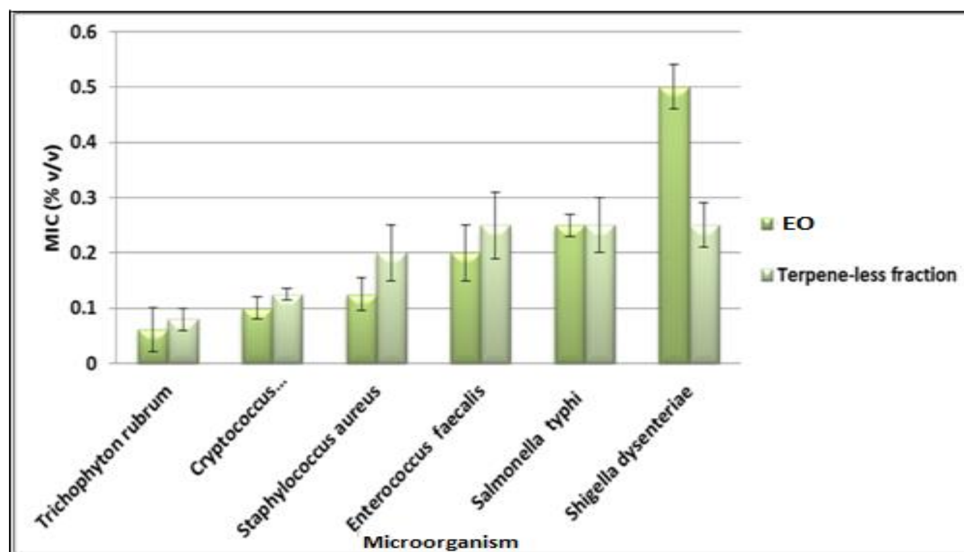


Fig 4: Antimicrobial activity demonstrated by *C. citratus* essential oil

A-*Trichophyton rubrum*; B-*Cryptococcus neoformans*; C-*Staphylococcus aureus*; D-*Enterococcus faecalis*; E-*Salmonella typhii*; F-*Shigella dysentriea*
[a-Total Essential Oil; b-Terpene-less fraction; c-Terpene-containing fraction; d-Positive control; e-Negative control]



Data analyzed by Tukey's HSD (P<0.05, n=5)

Fig 5: MIC of *C. citratus* essential oil against pathogenic strain

4. Conclusion

Cymbopogon citratus is unique in possessing essential oil as a source of wide array of terpenes of enormous International potential used in perfumery, flavouring and pharmaceutical preparations. The chromatographic method used for fractionating the essential oil was efficient to obtain terpene-less and terpene-containing components. The essential oil with the fractions exhibited good antioxidant and antimicrobial activity.

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