

Differential citral content of 15 lemongrass genotypes and their anti microbial property

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Abstract

In this present study antimicrobial activity of 15 promising cultivars of lemongrass were compared against 10 pathogenic microorganisms. Essential oil extracted from fresh leaves showed a distinctive variation in its oil percentage, citral content as well as antibacterial and antifungal potential against six bacterial and four fungal isolates. Direct correlation between citral content, a major constituent of the essential oil of lemongrass cultivars could not be established with their antimicrobial activity indices. MIC against *Escherichia coli* by plate dilution method was found to be very low (250 µg/ml). Inhibition zone diameter study of essential oils showed higher range of inter-specific variation within the cultivars. The cultivar OD19 with highest antibacterial index (Abl) 27.2 has the best potential to prevent bacterial growth, followed by Pragati and SD68 with Abl 24.66 and 24.02. RRL (B) 27 has got the lowest Abl of 11.4, thus found to be highly susceptible to majority of test bacteria. *Pseudomonas aeruginosa*, the most sensitive bacteria to lemongrass oil having Inhibition zone diameter (IZD) ≥90mm irrespective of type of cultivar. Similarly all 4 fungal test strains used in this study were found to be highly sensitive to this essential oil showing no visual growth in the Petri dish. Under these experimental conditions, the inter-specific variations in antibacterial potential of all fifteen distinct promising cultivars are more clearly evident but all genotypes possesses exceptionally good anti fungal property.

ABBREVIATIONS

AI- Antimicrobial Activity Index A_bI -Antibacterial Activity Index A_fI.- Antifungal Activity Index MIC-Minimum inhibitory concentration IZD-Inhibition zone diameter

INTRODUCTION

Lemongrass (*Cymbopogon flexuosus* Nees ex. Steud Wats) a perennial grass with lemony aura belongs to family Poaceae, though restricted in its distribution to selected patches of subtropical parts of Asia, Africa and America, has acclaimed significant global demand because of its varied range of applications in different industries viz; in perfumery and cosmetics for its lemon like scent, in food and beverage as flavoring and preservative agent, in agriculture as insect repellent, in soap, detergent and pharmaceuticals for its antimicrobial property, as well as in aroma therapy for its aromatic healing properties etc.^{1,2,3} . Although all these rare qualities are bestowed with lemongrass essential oil alone, development and release of new improved variety by traditional breeding has been very infrequent. Chemical characterization and biological property study to understand the genotypic variation concerning above desirable

properties, exists if any among cultivars released from different research stations of India, holds extremely significant. It is clear from the available literature that efforts are being taken by different groups of workers and of late some activity study has been reported on one/few released lemongrass varieties against one/few test organisms^{4,5,6,7,8} as well as on genetic diversity amongst the cultivars⁹, but neither concerted attempt has been taken to assay antimicrobial potential of many improved cultivars at large, screened against a group of microorganisms nor the effect of citral content on their antimicrobial activity has been reported.

Keeping the above facts in view, one such attempt has been taken in the present work, to establish a correlation between citral content and the antimicrobial property of essential oil extracts from 15 rare, promising, genetically stable cultivars released from different research stations of India against a host of 10 microbial isolates, pathogenic either to animals or plants and food spoiling microorganisms so that role of genetic make up of different cultivars on variation of antimicrobial property may be understood and future potential

drug molecules may be identified from this study for commercial exploitation.

MATERIALS AND METHODS

PLANT MATERIAL

The clumps from fifteen *Cymbopogon* cultivars were obtained from germplasm collection centre of Aromatic and Medicinal Plant Division, Regional Research Laboratory, Bhubaneswar, India where these varieties are domesticated, being collected from different research stations of India. The clumps obtained were planted and maintained in the herbal garden of Center of Biotechnology, Siksha 'O' Anusandhan University, Bhubaneswar to use its leaves for essential oil extraction. After 60 days of growth period the morphological variability was recorded in terms of plant height and number of tillers produced by all 15 cultivars namely Jorhat, Pragati, RRL (B) 2, SD 68, OD 19, OD 440, RRL (B) 1, RRL (B) 14, RRL (B) 16, RRL (B) 24, RRL (B) 26, RRL (B) 27, RRL (B) 28, RRL 16 and Jammu CK 5.

TEST ORGANISMS AND THEIR MAINTENANCE

Six bacterial (*Escherichia coli* MTCC1089, *Bacillus subtilis* MTCC 441, *Enterococcus faecalis* MTCC 2729, *Proteus mirabilis* MTCC 3310, *Pseudomonas aeruginosa* MTCC 647, *Staphylococcus aureus* MTCC3160) and four fungal (*Alternaria solani* MTCC 2101, *Aspergillus niger* MTCC 1344, *Helminthosporium solani* MTCC 2075, *Penicillium chrysogenum* MTCC 161) isolates obtained from Microbial type culture collection & gene bank (MTCC), Chandigarh, India were stored at -20°C. The above microorganisms were selected for assay study, as these are common pathogens either to plants or animals or causing food spoilage. The pure cultures were maintained by routine sub-culturing at one-week interval in nutrient agar and potato dextrose agar (Hi-Media laboratories private limited, Mumbai, India) slants for bacteria and fungi respectively.

EXTRACTION OF ESSENTIAL OIL

Essential oils from fresh, clean, weighed lemongrass leaves, extracted by hydro steam distillation using Clevenger's apparatus were collected and stored in sterile vials at -20°C. The extracted volume was recorded for calculation of percentage oil content with respect to the quantity of biomass used for particular cultivars.

GLC ANALYSIS/DETERMINATION OF CITRAL CONTENT

Citral analysis of the essential oil of lemon grass was performed by gas chromatography (GC). A microprocessor

based GC system (Chromatography and Instrument Co. Pune, India) fitted with a 51X3.2 mm stainless steel column packed with 10% carbowax treated with 20,000 molecular weight tetraphthalic acid (20 M+TPA) on Chromosorb W (WA) 80/100 mesh was used. Argon was used as the carrier gas at an inlet pressure of 110.4 KPa. The temperatures were programmed as follows: injector/detector 200-250°C and column was maintained isothermally at 115 °C. The essential oil extracted from fresh leaves of 15 cultivars was checked for citral content and the percentage was calculated.

Chemicals: Nutrient Agar (NA) and Potato Dextrose Agar (PDA) were used for cultivating microorganisms and dimethyl formamide (DMF) solvent was used for dilution of essential oil at a ratio of 1:3 of oil and DMF for proper spreading of oil on to the agar surface. All these chemicals and microbiological media were obtained from Hi Media Laboratory Private Limited, Mumbai, India.

INOCULUM PREPARATION

For evaluation of minimum inhibitory concentration (MIC) and inhibition zone diameter (IZD), inoculums from 10^{-1} dilution of 24 hours incubated sub-cultures were prepared from their freshly grown cultures of 10 different microorganisms. 0.5 ml. (10^{-6} CFU/ml of fungal or bacterial culture) of such diluted cultures was used as inoculums for all the experiments. The CFU/ml is determined by enumeration of viable bacterial cells and fungal conidia using standard plate count method taking 0.5 ml of 10^{-1} dilution from 24 hours old bacterial and 4-8 days old fungal culture tubes (which are regularly sub cultured at an interval of 15 days) and adjustment of the inoculums size was first done by making necessary dilutions after plate counting is done for these microorganisms so as to ensure the concentration of these organisms to contain approximately 1×10^{-6} CFU/ml in 0.5 ml of inoculums.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

Plate dilution method was followed to determine MIC of essential oil extract taking six different concentrations (100,150,250, 500,750µg/ml) against 0.5 ml of 10^{-1} inoculums dilution prepared from 24 hours incubated culture of *E.coli* into a sterile Petri plate followed by pouring 20ml autoclaved nutrient agar media so as to understand the minimum concentration needed to prevent the growth of the microbial strain and use the obtained MIC from this test for evaluation of inhibition zone diameter for all other extracts against ten test microorganisms. The seeded plates were

incubated at 37°C for 48 hours and the growth was noted down for different concentrations of extract separately. All the experiments were done in triplicates and positive, negatives controls were run parallel along with sample analysis.

The main objective of the present work being comparative evaluation of anti microbial potential of fifteen genotypes of essential oil extracts along with standard antibiotics by measuring diameter of inhibition zone ^{10,3}. MIC was obtained for all 15 extracts against a selected bacterial isolate *E. coli*. Available report concerning the effect of anti microbial property of lemongrass oil on growth inhibition of *E. coli* establishes the choice of such a strain for determination of MIC ^{6,11}.

ANTIMICROBIAL ASSAY

Lemongrass essential oil extracts were subjected to antimicrobial assay by Kirby-Bauer's method ¹² of measuring the diameter of zone of inhibition (IZD) using disc diffusion technique ^{13,14}. Nutrient agar and potato dextrose agar plates were prepared by pouring 20ml each in sterile Petri dishes for bacterial and fungal assay respectively and allowed to solidify. 0.5 ml of 10⁻¹ dilution of 24 hours old bacterial and 4-8 days old fungal cultures were used so as to ensure the concentration of these organisms to contain approximately 1X 10⁶ CFU/ml.

Sterilized cotton swabs dipped in respective cultures were swabbed on solidified agar surface. Pre-sterilized filter paper discs of 5mm diameter, which absorb 10-12µg sample/disc, were dipped into individual extract of 250µg/ml concentration separately and placed on the swabbed agar plates before incubation. Similar process is followed for controls using streptomycin and cotrimazole discs (10µg drug/disc), obtained from Hi-Media laboratories private limited, Mumbai, India as standard against bacteria and fungi respectively. Assay was also performed separately for evaluating the anti microbial activity of DMF, the solvent used to dilute the essential oil. At the end of incubation period, diameter of inhibition zones formed in all three replicates were measured in mm using measuring scale and the average of the three was determined ¹⁵.

ACTIVITY INDEX AND STATISTICAL ANALYSIS

Activity index (AI) of a particular cultivar was determined from the average of antibacterial index (A_bI) and antifungal index (A_fI). Antibacterial index (A_bI) and antifungal index (A_fI) for all cultivars were calculated as the mean value of

zone of inhibition against individual bacterial and fungal test strain respectively (Saikia et al., 2001). The correlation between Activity index (AI) and corresponding citral percentage for individual cultivars was established by statistical analysis through determination of coefficient of correlation *r* and *T* value from *T*-test.

RESULTS AND DISCUSSION

Comparative evaluation illustrates a higher range of phenotypic variation amongst 15 lemongrass cultivars while going through the morphological data, ascertaining an inverse relation between the height and number of clumps (table-1) ¹⁶. Notable variation in number of clumps from 10 to 26 with corresponding height of 134cms to 95cms respectively was found in varieties OD440 and RRL 16. From the Clevengers' extraction results as shown in table-1, RRL 16 variety was found to have highest percentage (1.12%) of oil and RRL (B) 27 was containing 0.36% of oil, the lowest among these cultivars.

Figure 1

Table 1: Phenotypic description, Essential Oil & Citral content of 15 Lemongrass Cultivars

Lemon grass variety	Plant Height (cm)	Tiller No./ Clump	Weight of leaves (gm)	Extracted Oil (ml)	Per centage (%)	Citral content (%)
Jorhat	135	22	111.44	0.6	0.58	88.53 ± 0.917
Pragati	130	18	170.41	1	0.58	86.62 ± 0.833
RRL (B) 2	126	24	151.47	0.6	0.39	87.98 ± 0.764
SD 68	120	14	80.89	0.4	0.49	88.6 ± 0.2
OD 19	130	22	29.55	0.2	0.67	89.15 ± 0.306
OD 440	134	10	62.3	0.3	0.48	87.15±0.416
RRL (B) 1	128	18	58.5	0.3	0.51	89.15±0.2
RRL (B) 14	120	21	53.65	0.3	0.55	90.58 ± 0.306
RRL (B) 16	110	20	78.7	0.6	0.76	90.27 ± 0.2
RRL (B) 24	122	22	73.64	0.5	0.67	88.38 ± 0.2
RRL (B) 26	132	24	52.8	0.5	0.94	92.04 ± 0.416
RRL (B) 27	132	12	27.2	0.1	0.36	86.27 ± 0.702
RRL (B) 28	122	22	28.46	0.2	0.7	89.69 ± 0.529
RRL 16	95	26	90	1	1.1	82.87 ± 0.702
JamunouCK 5	122	20	29.93	0.3	1	89.82 ± 0.416

Cultivar RRL (B) 26 was found to contain highest percentage of citral (92.04) and RRL 16 has got the lowest percentage (82.87) as found from in the GLC analysis report (table-1). When compared amongst the cultivars, citral content, acknowledged as the chief constituent of lemongrass oil, did not show any correlation at all with their

value of activity indices AI, AbI and Afi (denotes the antimicrobial potential the cultivar possesses) of the respective cultivars. The TC value of 0.87 (Calculated T value) calculated statistically from the coefficient of correlation ($r=0.235$) between the activity indices AI of individual cultivars and their corresponding percentage citral content, was found to be very much less than Tt value of 2.16 (tabulated T value) indicating that there exists no significant correlation between these two parameters which in turn establishes the fact that there exists no direct relationship between anti microbial potential and citral content.

Therefore, the antimicrobial properties of oil can not be attributed due to the presence of citral content in their oil alone (table-2) but may be the essential oil of cultivars in toto or citral in combination with other minor components. Wannissorn (1996) is also of similar opinion that lemongrass oil has greater activity than pure isolate of citral. Recent report of Saikia et al., (2001) on anti fungal activity of three genotypes of *Cymbopogon* sps. further supports the above idea that citral has lower activity comparable to the whole essential oil. Carriles et al., (2005) has demonstrated synergistic effects on fungi *Zygosaccharomyces bailii* inhibition when citral was used in combination with vanillin, thymol, carvacrol, or eugenol. All these above-mentioned information strengthen our interpretation that, the whole essential oil and not citral alone might be responsible for determining the antimicrobial potential of a cultivar irrespective of their citral content.

Figure 2

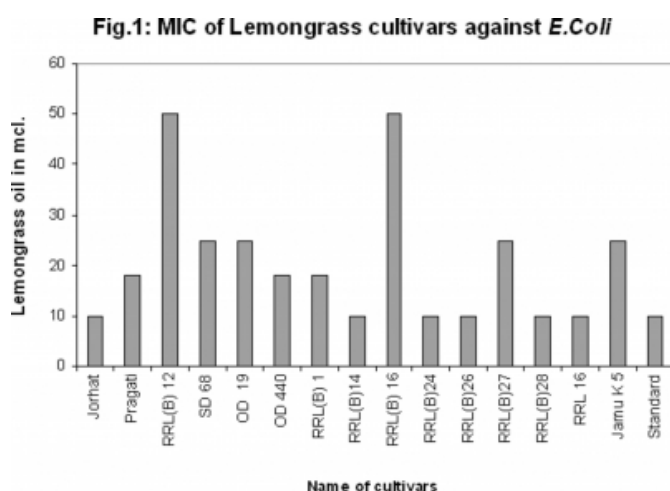
Table 2: IZD (mm), Antibacterial Index, Antifungal Index & Activity Index of 15 Lemmongrass Cultivars

Test Organism	Antibacterial Index						Antifungal Index				Activity Index	Citral content %		
	<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. mirabilis</i>	<i>E. faecalis</i>	<i>Paratyphosa</i>	<i>A. niger</i>	<i>H. solani</i>	<i>P. chrysogenum</i>					
Jorhat	11.6	31	28.6	18.3	21.6	90	90	90	90	40.28	90	65.1	38.5	
Pragati	19	33	28.3	23	20	90	90	90	90	42.66	90	66.3	36.6	
RRL (B) 2	16.6	30	26.6	19.6	14.6	90	90	90	90	39.4	90	64.7	38	
SD 68	24.6	29	33.3	18.3	15.3	90	90	90	90	42.02	90	66	38.6	
OD 19	32.2	28	30.3	23.6	22.3	90	90	90	90	45.2	90	67.6	39.2	
OD 440	25.3	30	25.6	13.6	14	90	90	90	90	39.7	90	64.9	37.2	
RRL (B) 1	11.3	30	30.3	6.6	18.6	90	90	90	90	37.36	90	63.7	39.2	
RRL (B) 14	7	29	26.3	28	21.6	90	90	90	90	40.38	90	65.2	30.6	
RRL (B) 16	9.6	32	25	20.3	18	90	90	90	90	38.9	90	64.5	30.3	
RRL (B) 24	11.3	26	32.2	23.3	19.6	90	90	90	90	40.4	90	65.2	38.4	
RRL (B) 26	13	26	33	15.6	21.3	90	90	90	90	39.78	90	64.9	32	
RRL (B) 27	6	7.3	18.3	15.3	8.3	90	90	90	90	27.38	90	58.7	36.3	
RRL (B) 28	6.6	29	30	15.3	12.3	90	90	90	90	34.18	90	62.1	39.7	
RRL 16	11.3	30	24.6	11.6	18.3	90	90	90	90	37.22	90	63.6	32.9	
Jamu CK 5	14.3	29	26	16.6	12.6	90	90	90	90	37.62	90	63.8	39.8	
Standard	19.2	31	29.6	2.8	25.4	31.4	11	27.8	11.8	23.8	23.22	19	20.9	-

250µg/ml of essential oil or some times less than that (100µg/ml, 180µg/ml) was found to be sufficient enough to inhibit the growth of *Escherichia coli* by all the cultivars except only RRL (B) 2 and RRL (B) 16 as shown in fig.-1. This indicates that very small quantity of oil is needed for effective growth inhibition and hence for further IZD determination, this concentration of 250µg/ml was considered optimum. Hammer et al. (1999) have reported about effectiveness of lower concentration of essential oil (0.06%) in inhibiting microbial growth from different plant sources including *Cymbopogon* sps against *E. coli*.

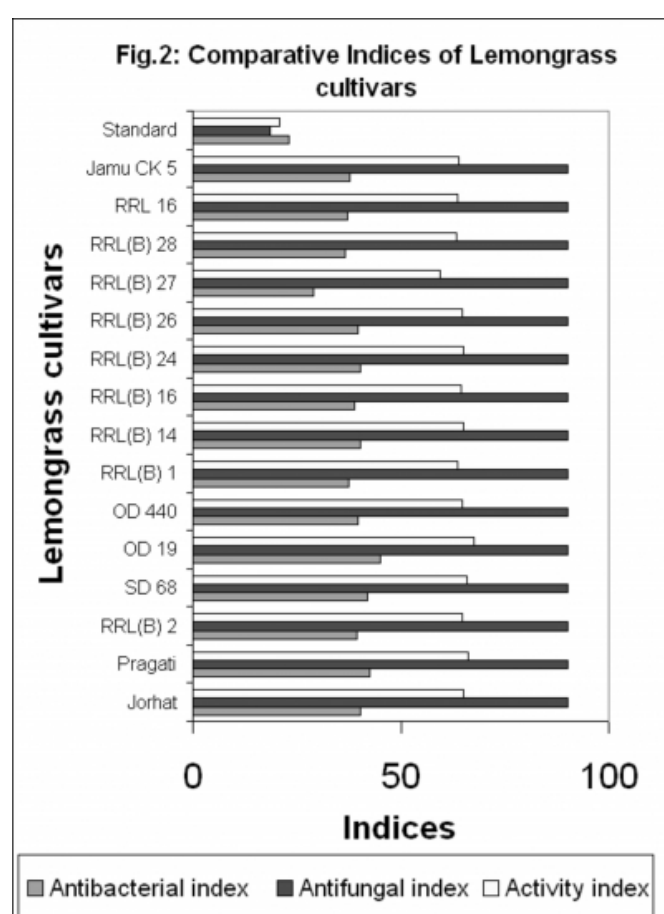
The highest growth inhibiting potential was exhibited by the essential oil from cultivars SD68 (33.3mm IZD), Pragati (33mm IZD), OD19 (32.2mm, 22.3mm IZD) and RRL (B) 14 (28mm IZD) against test organisms *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Proteus mirabilis* respectively whereas essential oil of cultivar RRL (B) 27 was found to be least anti-microbial ($A_0I-29.04$) cultivar having the least IZD against majority test microorganisms *Bacillus subtilis* (18.3mm IZD),

Figure 3



Enterococcus faecalis (8.3mm IZD), *Staphylococcus aureus* (7.3mm IZD), *Escherichia coli* (6mm IZD). This indicates that cultivar RRL (B) 27 possesses least anti microbial properties and cultivars SD68, Pragati and OD19 were found to be more anti-microbial with better potential to prevent different strains of test bacteria used for this study (table-2). Similarly from the anti bacterial index A_bI (fig-2) we can infer that cultivar OD19 with highest A_bI (45.2) has the best potential to prevent bacterial growth, followed by Pragati and SD68 with A_bI (42.66 and 42.02) but RRL (B) 27 with the lowest A_bI of 11.4 was found to possess very less anti-microbial property against majority of test bacteria. Baratta et al. (1998) in his study reported similarly the significant amount of anti bacterial activity possessed by the volatile oil of lemongrass along with other plants against few pathogens.

Figure 4



During determination of IZD an exceptionally high IZD of 90mm was observed against *Pseudomonas aeruginosa* by all 15 cultivars (complete haloing of Petri plates with no visual colony or growth although positive control showed characteristic growth on Petri plates). This necessitated a cross verification and reestablishment of the result, for which 24 hours incubated *Pseudomonas aeruginosa* seeded Petri plate was treated with an oil soaked paper disc onto its centre which after a period of 24 hours reaction time showed no visible bacterial growth. This confirms not only the high inhibiting potential of the oil but also the bactericidal property it possesses. Similar reports of complete haloing of Petri plates with IZD > 90mm were found where *Vibrio cholerae* isolates were treated with lemongrass essential oil (Pattnaik et al., 1995)

IZD study of all these essential oils against 4 fungal pathogens showed no visible fungal growth in any of the Petri plates (90mm) with high anti fungal index (A_fI) indicating higher level of broad spectrum antifungal property of essential oil from all the cultivars with respect to the tested strains *Alternaria solani*, *Aspergillus niger*, *Helminthosporium solani*, *Penicillium chrysogenum*. This

result not only gives us information about the broad spectrum antifungal potential of these cultivars but also ascertain that genotypic variation does not exist with respect to antifungal activity under these testing conditions or may be the essential oil has got very excellent anti fungal activity at this oil concentration which is more than adequate to bring about inhibition even by the variety most susceptible to bacteria (RRL (B) 27 against *E. coli*, *S. aureus*, *B. subtilis* and *E. faecalis* showing lowest A_bI).

This is clearly evident from the table-2 that there lies a huge range of variation in the potentiality among different cultivars in preventing the growth of individual test organisms which means that genetic make up within a species like *Cymbopogon flexuosus* determines the range of activity a cultivar exhibits. Pattanaik et al., (1995) are of similar opinion that the influence of genetic difference of *Cymbopogon* sps. exists on the antibacterial activity of their essential oil.

Above all, when compared with the activity of standard antimicrobial agent streptomycin and cotrimazole assessed against the test bacterial and fungal isolates, lemongrass essential oils of many cultivars were found to encompass little higher activity against individual bacteria and excellent multifold (3 or more times) activity against all four fungal isolates as well as one bacterial isolate, *Pseudomonas* sps.

The indices AI , A_bI and A_fI indicate comparative activity profile specific-to-specific cultivars against a host of bacteria and/or fungus. This means that there lies a distinctive inter-specific variation in antimicrobial activity within all 15 cultivars of lemongrass used for analysis. Specific genotypes have distinct variation not only in number of clumps or height or total essential oil content and citral percentage but also in anti microbial and antifungal activity profile.

CONCLUSION

The analysis of 15 cultivars of lemongrass for their essential oil, citral content and anti microbial activity can be summarized as below. The percentage essential oil and citral content of different cultivars has got no visible correlation with their antimicrobial properties.

Though wide range of variability exists amongst the essential oil of lemongrass cultivars with respect to its growth inhibiting capacity against several pathogens, still there lies some specificity of cultivars in their antibacterial activity towards test microorganisms like SD68 against *Bacillus subtilis*, Pragati against *Staphylococcus aureus*,

RRL (B) 14 against *Proteus mirabilis*, OD19 against *Escherichia coli* and *Enterococcus faecalis*.

Therefore, essential oils of these cultivars after further analytical and pharmacological study may be recommended to use as a new potential drug molecule as antibacterial, antifungal agents or may be both. Similarly cultivar RRL (B) 16 containing highest percentage oil content having excellent potential to prevent growth against *Pseudomonas aeruginosa* may be recommended to use against its infection as bio-bactericide in crop production especially in organic cultivation after commercial feasibility study.

Another attempt may be taken to develop a formulation of lemongrass essential oil extract to be used as a suitable bio-fungicide against plant infections caused due to major plant pathogens like *Helminthosporium* sps and *Alternaria* sps as well as to be used against animal pathogens like *Aspergillus niger* and *Penicillium chrysogenum* for their extraordinary anti fungal properties.

Thinking another step forward a combination of essential oil from OD19 with either SD68 and/or Pragati may be used as a future broad spectrum antibacterial and/or antifungal candidate after investigating its synergistic effect exists if any (such study is in progress in this laboratory).

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