In Vitro Antimicrobial Study of Plant Essential Oils and Extracts

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Citation

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Abstract

Present investigation provides comprehensive and quantifiable information on in vitro assay of 31 medicinal plant oils and extracts exposed to ten pathogenic and food spoiling microorganisms by agar diffusion method through determination of inhibition zone diameter. Among the bacterial species exposed to O. sanctum oil, highest susceptibility was displayed by Enterococcus faecalis whereas to lemongrass oil the highest was shown by Pseudomonas aeruginosa as denoted by SI, the susceptibility index. Little variation in activity has been observed from the anti bacterial index (A_bI) among lemongrass cultivars. Highest growth inhibiting potential was shown by lemongrass cultivar CF4 against pathogen Bacillus subtilis and food poisoning organism Staphylococcus aureus and by cultivar CF5 against human pathogens Escherichia coli, Enterococcus faecalis. Only specific plant chemical extracts like acetone extract of P.niruri, methanol extract of stevia demonstrated activity against plant pathogens A. solani and P. aeruginosa respectively but acetone extract of M. coenigii has significant anti microbial activity against animal pathogen P. mirabilis, E. feacalis. Amongst the essential oil exposed to four fungal pathogens A. niger, P. chrysogenum, A. solani and H. solani, excellent anti fungal activity was observed by all lemongrass cultivars followed by C. longa and O. sanctum which is clearly evident from anti fungal index (A_fI) but among the chemical extracts tested only petroleum ether extract of stevia and ethanol extract of P.niruri were found to have optimum activity respectively against A. solani and H.solani . Water and chloroform extract of P.niruri, ethanol and cyclohexane extract of Stevia, water extract of M. coenigii shows no or meager activity.

INTRODUCTION

Extracts of plant origin have been known to possess many therapeutic properties since thousands of years [1]. Use of plant parts as folklore medicine have been trailed by traditional healers since time immemorial though scientific basis of their antimicrobial actions, specificity in performance against microbial strains, role of active ingredients or major constituents of their products, application dosage range and toxic effects have only been understood in recent times after plant essential oils and extracts have been subjected to various types of analytical studies with the advent of sophisticated instruments as well as development of newer methodologies. With time these oils and extracts have been understood to encompass the attributes accounted not only for their fragrance and flavor [2] but also for their antimicrobial nature for treating plant animal and human diseases [3-6] and food preservative properties [7]. Consequently attempts have been taken by different workers on in vitro activity study of few oils and extracts [15-18] using numerous plant from many sources discretely, following variable methodologies against varied microbial isolates but majority of available reports are dealt on essential oils/plant extracts of one/few plants against one/few organisms by using diverse protocols like pour well, pour disc, swab disc etc. by means of determining minimum inhibitory concentration or diameter of inhibition zone [19-22]. As there exists a lot of variations in parameters involved in above assay of anti microbial study and even though the available data are useful, these are not directly comparable to each other and no general interpretation can be established [23]. Moreover, there has been resurgence of interest in quest of newer molecules with anti microbial property of biological origin among scientific community due to development of resistance by pathogens [8], expensive treatment regimen of synthetic drugs already in practice and their gross side effects due to indiscriminate use [9-14]. Keeping the above facts in view the present investigation was carried out to evaluate 31 extract types from 6 plant sources against a host of 10 microbial strains (two gram positive, four gram negative bacterial and four fungal), pathogenic either to plants, animals and/or human beings or causing food spoilage, so as to establish a comparative data base of these extracts

analyzed by following single standard recommended protocol as well as to identify few cheap source of future potential candidates of plant origin against disease causing pathogens.

MATERIALS & METHODS

PLANT MATERIAL

Fresh leaves of Stevia rebeudiana (stevia) were collected from Silviculture station, Department of Forest, Bhubaneswar, Orissa. Ocimum sanctum (tulsi), Murraya coenigi (curry leaves) and Phyllanthus niruri (Bhoomi amalaki) leaves were collected from the botanical garden of Regional Plant Resource Centre, Bhubaneswar and Curcuma longa (turmeric) rhizome was obtained from the herbal garden plants of Center of Biotechnology, Siksha 'O' Anushandhan Uinversity, Bhubaneswar for this present work. The slips of 10 Cymbopogon flexousus (lemongrass) cultivars namely Jorhat-CF1, Pragati-CF2, RRL (B)2-CF3, SD 68-CF4, OD 19-CF5, OD 440-CF6, RRL (B) 24-CF7, RRL (B) 26-CF8, RRL (B) 27-CF9 and RRL (B) 28-CF10 (these stable, elite cultivars are released from different research stations of India) were obtained from germplasm collection centre of Aromatic and Medicinal Plant Division, Regional Research Laboratory, Bhubaneswar.

TEST ORGANISMS AND THEIR MAINTENANCE

Six bacterial and four fungal pure cultures (Escherichia coli MTCC1089, Bacillus subtilis MTCC441, Enterococcus faecalis MTCC2729, Proteus mirabilis MTCC3310, Pseudomonas aeruginosa MTCC 647, Staphylococcus aureus MTCC3160, Alternaria solani MTCC2101, Aspergillus niger MTCC1344, Helminthosporium solani MTCC2075, Penicillium chrysogenum MTCC161) obtained from Microbial type culture collection & gene bank (MTCC), Chandigarh, India were stored at -20ºC. These microorganisms were selected for microbial assay study, as these are common pathogens either to plants or animals or food spoiling microbes. The pure cultures were maintained by routine sub-culturing at one week interval in Muller Hilton agar and Sabroud 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 leaves of tulsi, lemongrass, curry leaves and rhizome of turmeric extracted by hydro steam distillation using Clevenger's apparatus were collected and stored in sterile vials.

SUCCESSIVE SOLVENT EXTRACTION

Successive solvent extraction was performed for Stevia rebeudiana, Murraya coenigi and Phyllanthus niruri. Leaves were washed, air dried for 7-8 days, ground into powder before putting into the flask of Soxhelet apparatus for extraction using six different solvents viz; petroleum ether, benzene, chloroform, acetone, ethanol, water with increasing order of their polarity to extract the phyto-constituents (all solvents used were HPLC grade obtained from Hi Media laboratories private limited, Mumbai, India) separately at temperature 20 ºC for 3-4 hours except in case of chloroform extraction where the leaf sample was submerged in 10% chloroform for 2-3 days at room temperature. The extracts were filtered using Whatman No.1 filter paper. All the filtrates were then evaporated to dryness under reduced pressure and stored in labeled sterile screw capped bottles at -20 ºC until further analysis.

CHEMICALS

Muller Hilton agar (MHA) and Sabraud dextrose agar (SDA) were used for cultivating microorganisms and Dimethyl formamide (DMF) solvent was used for dilution of essential oil. All these chemicals were obtained from Hi Media laboratories private limited, Mumbai, India.

INOCULUM PREPARATION

For evaluation of inhibition zone diameter (IZD), inoculum from 10^{-1} dilution of 24 hours incubated sub-cultures for bacteria and 4-8 days incubated sub-cultures of fungi were prepared from their freshly grown cultures of 10 different microorganisms. 0.5 ml. (equivalent to 10^6 CFU/ml of fungal spore or bacterial cell) of such diluted cultures was used 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 1X 10^6 CFU/ml in 0.5ml of inoculums.

MICROBIAL ASSAY STUDY

IZD was determined by disc diffusion method (24) by placing 5mm diameter Whatman's No.1 filter paper disc dipped in essential oil onto microorganism inoculated solidified media. Anti microbial property of standard antibiotic discs impregnated each with 10 g of either streptomycin or cotrimazole used against bacteria and fungi respectively, were evaluated against all the extracts. The antibiotic discs were obtained from Hi Media laboratories private limited, Mumbai, India. Three such replicates were incubated at 27ºC for 48-72 hours for fungi and 37ºC for

24-48 hours for bacteria to evaluate the zone diameter in mm. The average of three measurements was recorded. For all above experiments one positive and one negative control were run parallel. Dimethyl formamide solvent used to dilute essential oil and six chemical solvents used for successive extraction were examined for having any antimicrobial property.

ACTIVITY INDEX

Antibacterial index (A_bI) and antifungal index (A_fI) for each extract type were calculated as the average value of zone of inhibition against a group of gram +ve and gram -ve bacterial and fungal test strains respectively and the average of Antibacterial index (A_bI) and antifungal index (A_fI) gives total Activity index (AI) of a particular cultivar. Susceptibility index (SI) denotes the range of susceptibility of any microorganism against a range of extract(s) of a plant that is calculated from the average of antimicrobial activity (IZD) obtained against an individual microbial isolate.

RESULTS AND DISCUSSION

It is clear from figure 1 that all extract types respond almost equally towards gram +ve and gram –ve bacteria except lemongrass essential oil and acetone extract of P. niruri which shows better anti bacterial property towards gram –ve bacteria than gram +ve ones and reverse response was observed against benzene extract of M. coenigii.

Lower susceptibility index (SI<11mm) exhibited by chemical extracts of P. niruri indicates its ineffectiveness against almost all microbial isolates to inhibit their growth except acetone which shows excellent activity against A. solani (IZD27mm), greater than that of the standard antibiotic (fig 4). Activity at par with standard was observed on exposure to bacterial isolate E.faecalis (IZD18mm) and fungal pathogen H.solani (IZD12mm). Ethanol extract showed activity comparable to standard against A. solani and H.solani. Chloroform and water extracts demonstrated no activity at all (fig 3).

Stevia also exhibited lower susceptibility index (SI<10mm) but it is clearly depicted in figure-4 that among the bacterial pathogens selected for this study, highest susceptibility (SI9.27mm) was exhibited by S. aureus invariably by all four extracts, petroleum ether, cyclohexane, chloroform, water extracts but no activity was reported by acetone and ethanol extract (table 1). The water extract has got the antimicrobial activity only against E. coli, S. aureus and B subtilis (fig 1). Similar results were reported by M.B. Tadhani and R. Subhash (2006) [7] that water extracts of Stevia leaf showed activity against B. subtilis and S. aureus only. IZD obtained by the ethanol extract in the present study also coincides with the response got by same workers using methanol extract which gave the highest zone of inhibition against P. aeruginosa and minimum against S. aureus . Acetone, chloroform, cyclohexane showed less activity but petroleum ether exhibited the total best antimicrobial property against all six bacterial species (AI-12.8).

As shown in figure 2 the highest anti fungal index (A_fI-15) for stevia was found in case of petroleum ether extract irrespective of 4 fungal species used, namely A. niger, P. chrysogenum, A. solani and H. solani as depicted in table 1.

Figure 1

Table.1, IZD of essential oils and extracts in mm (includes disc diameter)

Serial Number (1-31)	Name of plants	Solvents used	Extract type	Plant parts	Gram + ve		Gram–ve Strains				Fungal Stains			
					S. aureus	*B. subtilis*	*E. coli*	*P. mirabilis*	*E. faecalis*	*P. aeruginosa*	*A. solani*	*A. niger*	*H. solani*	*P. chrysogenum*
1	*Phyllanthus niruri*	PE	CE	L	8.7	7	9	7	9	8.33	7	12	5	5
2		BE	CE	L	7.7	7.3	10	5	8.7	5	8	5	9	9
3		CH	CE	L	5	5	5	5	5	5	5	5	5	5
4		AC	CE	L	10	15	21	13	18	18.3	27	5	9	5
5		ET	CE	L	14	17	10	13	13	16.6	12	5	12	12
6		WT	CE	L	5	5	5	5	5	5	5	5	5	5
7	*Stevia rebaudiana*	PE	CE	L	16	10	7	10	13	11	16	16	14	14
8		CY	CE	L	9	5	5	5	6	7	5	5	5	8
9		CH	CE	L	11	5	7	7	5	5	5	5	9	7
10		AC	CE	L	5	10	10	5	8.3	6	7	5	5	5
11		ET	CE	L	5	5	5	11	5	8	5	9	5	5
12		WT	CE	L	9.3	9	11	5	5	5	6	11	8	10
13	*Murraya coenigii*	PE	CE	L	7	7.6	9	5	8.9	10.6	7	8	9	7
14		BE	CE	L	24	6	10	6	5	9.6	8	9	10	9
15		CH	CE	L	9.6	5	5	5	8	5	7	5	5	9
16		AC	CE	L	21	16	20	26	21	15.7	6	10	5	5
17		ET	CE	L	10	5	12	25	6	5	5	5	5	10
18		WT	CE	L	5	5	5	5	5	5	5	5	5	5
19	*Cymbopogon flexuosus (lemongrass) Cultivars*	CF1	EO	S	31	29	12	18	22	90	90	90	90	90
20		CF2	EO	S	33	28	19	23	20	90	90	90	90	90
21		CF3	EO	S	30	27	17	20	15	90	90	90	90	90
22		CF4	EO	S	29	33	25	18	15	90	90	90	90	90
23		CF5	EO	S	28	30	32	24	22	90	90	90	90	90
24		CF6	EO	S	30	26	25	14	14	90	90	90	90	90
25		CF7	EO	S	26	32	11	23	20	90	90	90	90	90
26		CF8	EO	S	26	33	13	16	21	90	90	90	90	90
27		CF9	EO	S	7.3	18	6	15	8.3	90	90	90	90	90
28		CF10	EO	S	29	30	7	15	12	90	90	90	90	90
29	*M. coenigii*		EO	L	16	5	5	8	10	12	7	9	8	5
30	*O. sanctum*		EO	L	16	19	9	7	20	18.6	12	25	37	23
31	*C. longa*		EO	R	19	14	11	19	18	17.3	12	12	90	12
Standard antimicrobial discs					31	30.5	25	28	19	28.25	11	28	12	24
Susceptibility Index					28	28	15	18	17	31.4	90	90	90	51
DMF & Other Solvents					5	5	5	5	5	5	5	5	5	5

PE=Petroleum ether, CY=Cyclohexane, BE=Benzene, CH=Chloroform, AC=Acetone, ET=Ethanol, WT=Water, CE=Chemical extract, EO=Essential oil, L=Leaves, R=Rhizome, S=Slips, (IZD 5 mm - no activity)

M. coenigii showed no activity. Volatile essential oil of M. coenigii demonstrated some activity when exposed to S. aureus only (IZD The susceptibility index SI 13.2 of M. coenigii for S. aureus indicates higher susceptibility of the

organisms towards it as compared to those of Phylanthus species and Stevia sps (fig 4). Acetone was found to possess highest A_bI with IZD26 mm against P. mirabilis parallel to that possessed by standard and an IZD of 21.3mm as in table 1. Activity above the standard was observed against E. feacalis. Water extract of 15.6mm).

In figure 3 the anti microbial index for essential oil of O. sanctum has shown to be higher than that of the standard and having equivalent or higher IZD against bacterial isolate E.faecalis and all fungal isolates A. solani, A. niger, H. solani and P. chrysogenum with anti fungal index (A_fI-24) as given figure 2. H.solani was found to be most susceptible to oil of ocimum forming an IZD of 37mm. AI-32 of C. longa essential oil was found to be higher than that of the standard as well as of all other plant extracts except lemongrass oil. IZD 18mm equivalent to that of standard against bacteria E.faecalis and IZD 12mm, IZD 90mm above the IZD of standard against A. solani and H. solani respectively exhibited by oil extract of C. longa. It has higher A_fI than other extracts except lemongrass.

Highest range of susceptibility index (SI) was exhibited by almost all microbial isolates exposed to essential oil of 10 lemongrass cultivars establishing lemongrass proving to be the best antimicrobial potential among the plant extracts tested. Highest value of all indices (A_fI, A_bI & AI) also supports the above statement, which is clearly evident in all the figures (fig 1,2,3,4).

Figure 2

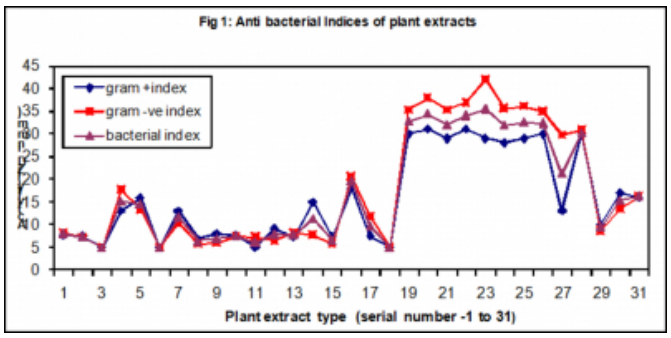


Figure 3

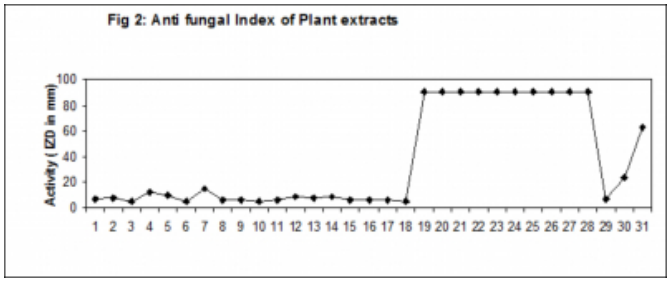


Figure 4

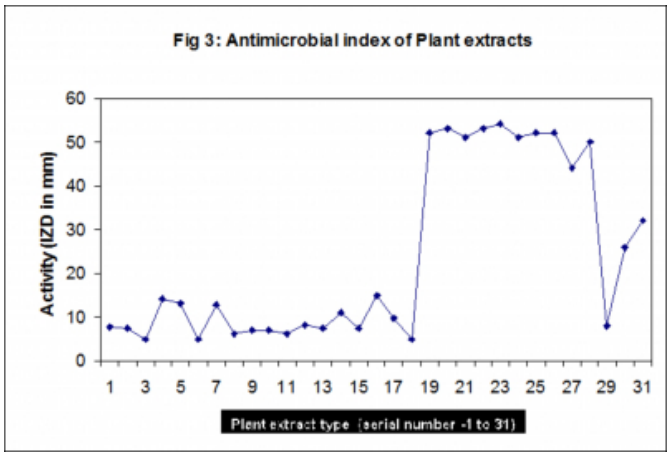
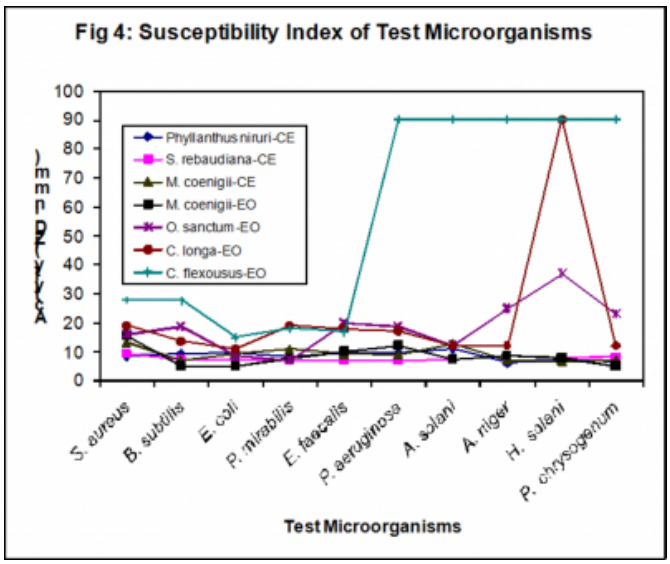


Figure 5



M. coenigii showed no activity. Volatile essential oil of M. coenigii demonstrated some activity when exposed to S. aureus only (IZD The susceptibility index SI 13.2 of M. coenigii for S. aureus indicates higher susceptibility of the organisms towards it as compared to those of Phylanthus species and Stevia sps (fig 4). Acetone was found to possess highest A_bI with IZD26 mm against P. mirabilis parallel to

that possessed by standard and an IZD of 21.3mm as in table 1. Activity above the standard was observed against E. feacalis. Water extract of 15.6mm).

However, there lies a lot of variation in its activity with respect to cultivar types and microorganisms as well as certain range of specificity is also exhibited towards few isolates. The highest growth inhibiting potential was exhibited by the essential oil from cultivars CF4 and CF5 against test organisms Bacillus subtilis, Staphylococcus aureus and Escherichia coli, Enterococcus faecalis, respectively whereas essential oil of cultivar CF9 was found to have the least IZD against majority test microorganisms Bacillus subtilis (18.3mm IZD), Enterococcus faecalis (8.3mmIZD), Staphylococcus aureus (7.3mm IZD), Escherichia coli (6mm IZD) as in table 1. This indicates that cultivar CF9 possesses least anti microbial properties and cultivars CF4, CF2and CF5 were supposed to have better potential to prevent different strains of test bacteria used for this study (table-1). Interpretation can be derived from fig 1 that the anti bacterial index A_bI that cultivar CF5 with highest A_bI (27.2) has the best potential to prevent bacterial growth, followed by CF2and CF4 with A_bI (24.66,24.02) but CF9 has got the lowest A_bI of 11.4, found to be the highly susceptible to majority of test bacteria. Baratta et al. (1998) (4) in his study reported significant activity against few pathogens by the volatile oil of lemongrass and other plants. During determination of IZD an exceptionally high IZD of 90mm was observed against Pseudomonas aeruginosa by all 15 cultivars (complete haloing of petriplates with no visual colony or growth though positive control showed characteristic growth on petriplates). Similar report was found with IZD > 90mm using essential oil of lemongrass against Vibrio cholerae [25]. IZD study of all these essential oils against 4 fungal pathogens showed no visible fungal growth in any of the petriplates (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. When compared with activity index (AI) of standard, (AI) of lemongrass cultivars were found to possess 3 or more times potent antimicrobial properties. (S. Pattanaik et al.1995) [25] have reported the influence of genetic difference of Cymbopogan sps. on the antibacterial activity of their essential oil.

CONCLUSION

The present study has demonstrated that volatile essential oil extract of O. sanctum has considerable anti bacterial potential against animal pathogen E.faecalis. Lemongrass oil has got the best anti bacterial property amongst all oil tested but little variation in activity has been observed at cultivar level with highest growth inhibiting potential by cultivars CF4 against pathogen Bacillus subtilis, food poisoning organism Staphylococcus aureus and CF5 against human pathogens Escherichia coli, Enterococcus faecalis respectively. Exceptionally significant growth inhibiting property was observed with lemongrass essential oil exposed to the pathogenic bacteria Pseudomonas sps. Only specific plant chemical extracts like acetone extract of P.niruri, methanol extract of stevia and acetone extract of M. coenigii have significant anti microbial activity specifically against plant pathogens A. solani and P. aeruginosa and animal pathogen P. mirabilis, E. feacalis respectively

Essential oil of majority of plants exposed to four food spoiling and plant disease causing fungal pathogens A. niger, P. chrysogenum, A. solani and H. solani showed excellent anti fungal activity lead by all lemongrass cultivars followed by C. longa and O. sanctum but among the chemical extracts tested petroleum ether extract of stevia and ethanol extract of P.niruri found to have optimum activity against A. solani and H.solani only.

The above screening results enumerates the existing potential of plant chemical extracts and essential oils to be used as suitable candidate as medicine, pharmaceuticals and food preservatives of plant origin for treating plant and animal disease causing pathogens and food spoiling microorganisms so that a safe alternate to existing chemical at low cost may be identified. However, for whatever purpose it may be used, issue of safety and toxicity needs to be addressed following recommended in vivo studies to confirm the validity of these results.

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