Asian Journal of Biological and Life Sciences | Original Research

Antimicrobial activity of *Cymbopogon Species* against human pathogens and molecular characterization by RAPD markers

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Submitted : 26.11.2012 Accepted :19.01.2013 Published : 30.04.2013

Abstract

Cymbopogon is a tall perennial grass and shows a great potential for antimicrobial compound against microorganisms. In this study the extracts of three different species of *Cymbopogon* (*C. martinii, C. citratus* and *C. nardus*) was tested against four pathogens (*Salmonella sp., Klebsiella sp., Staphylococcus sp.* and *Enterobacter sp.*). All the pathogenic microorganisms were isolated from clinical samples and identified by biochemical characterization. Organic solvent like Ethanol, Methanol, Acetone, Chloroform and Diethyl-ether was used for the extraction. Antimicrobial assay was done by agar well diffusion method on Mueller-Hinton Agar plates. The extracts of *Cymbopogon* species were effective against all four pathogens, and *C. citratus* showed maximum antibacterial activity than other species. Also, *Cymbopogon* potency was enhanced by methanol extraction which indicates that active material was extracted well in methanol compared to other solvents. These results imply that these medicinal plants can be used as a source of novel drugs for the treatment of infectious diseases caused by pathogenic microorganisms. These three selected *Cymbopogon* species were characterized by RAPD markers. Dendrogram constructed by cluster analysis of RAPD markers showed that *C. martinii* and *C. citratus* are closely related. Since morphological differences among these species are indistinctive, RAPD characterization can be helpful in their Discrimination. Also knowledge of genetic diversity can be used to identify the specific antimicrobial agent in these Cymbopogon species.

INTRODUCTION

Medicinal plants cure number of diseases which guide for investigation of medicinal plants as potential sources of new bioactive and antimicrobial agents [1, 2]. India has a very rich botanical wealth and thousands of species are used in different parts of country since ancient times to cure specific malady and shown great medicinal and economical value [3]. Earlier reports already demonstrated the antimicrobial activity of different herbs available in India [4, 5]. *Cymbopogon* is a tall perennial grass, thought to have origin in Malaysia and found growing in India and other parts of South East Asia [6]. Chemical composition of the essential oil of *Cymbopogon* varies according to the geographical origin and their main constituent are alkaloids, saponin, asitosterol, terpenes, alcohols, ketone, flavonoids, chlorogenic acid, caffeic acid, p-coumaric acid and sugars [7, 8]. *Cymbopogon* is thought to have anti-tussis, analgesic, anti-hermetic, antithermic, anticardiopatic, antispasmodic, antiallergic and anti-inflammatory action [9]. It is already reported that *C. citratus* has antimicrobial and antioxidant activity and most of the earlier report showed that "Citral" is responsible for its activity [10].

Although, number of new antimicrobial drugs is being invented, bacteria acquire resistance to these drugs; this creates necessity for finding new antimicrobial drugs [11]. Different methods can be used for extraction of antimicrobial compound such as solvent extraction which involves separation of the constituents from the mixture using a volatile solvent. The desired constituent gets separated in solvent due to the strong affinity for them and then molecules are removed by distillation [12]. In this study the extracts of three different species of *Cymbopogon* (*C. martinii, C. citratus, C. Nardus)* was tested for antimicrobial activity against four pathogens (*Salmonella sp., Klebsiella sp., Staphylococcus sp.* and *Enterobacter sp.*).

Cymbopogon species show variation in morphological trait and essential oil composition at inter- and intra species levels as they are influenced by environmental conditions [13-15]. Comparative genetic analyses have shown that different plant species share orthologous genes for similar function and have highly conserved gene content. Due to these fluctuations in morphological traits and conserved gene content molecular markers such as RAPD are often used to determine genetic diversity amongst the cultivars [15]. Knowledge of genetic diversity can be applied for plant conservation and improvement; therefore an attempt was made in determining the genetic diversity in *Cymbopogon* species. In the present study genetic diversity was determined by RAPD technique in three selected cultivars *C. martinii, C. citratus and C. Nardus* having antibacterial activity.

MATERIALS AND METHODS

Sample collection and Solvent extraction

Medicinal plants such as *Cymbopogon citratus, Cymbopogon martini* and *Cymbopogon Nardus* were collected from University of agriculture, Bangalore. Plant leaves were dried under shade and dried leaves were crushed using mortar and pestle. Then 5 g of crushed plant material was kept on rotary shaker along with 50 ml of different solvents like Ethanol, Methanol, Acetone, Chloroform and Diethyl ether for 2 days. The extract was concentrated by solvent evaporation and used for antimicrobial activity.

Isolation of the microorganisms

The pathogenic microorganism were isolated from clinical samples procured from diagnostic/pathology labs in Bangalore and identified by Gram staining and biochemical characterization. The isolated microorganisms were found to be *Salmonella sp., Klebsiella sp., Staphylococcus sp.* and *Enterobacter sp.*

Determination of antimicrobial activity

The antimicrobial activity was determined by the well-diffusion method. Overnight grown bacterial cultures from liquid nutrient broth media was transferred to sterile Petri plate with Mulleher Hinton (MH) agar medium (HiMedia Laboratories Limited, Mumbai, India) and was spread with sterile spreader to create a lawn. Wells of 6 mm were punched into the previously seeded MH agar plates using sterile cork borer. About 50 μl of the different solvent extract was placed in the wells and allowed to diffuse for 2 h at 4^0C and then the plates were incubated at 37°C for 24 hrs. The activity was determined by measuring the diameter of the inhibition zones around each well and expressed in millimeter.

Determination of minimal inhibitory concentration (MIC)

The extracts that exhibited considerable activity initially were further used for MIC determination. The extracts of the test samples were tested in four dose levels of 10 µl, 20 µl, 30 µl and 40 µl. The overnight grown bacterial culture was transferred on MH agar plate and wells were punched out using a sterile 6 mm cork borer. Different concentration (1040 µl) of the extract was placed in separate well allowed to diffuse for 2 h at 4^0C and then the plates were incubated at 37°C for 24 hrs. The zone of inhibition was observed and the lowest amount of the test sample showing zone of inhibition was recorded as the MIC.

DNA Isolation and PCR Amplification:

DNA was isolated from fresh leaves as per the procedure described previously [16]. The polymerase chain reaction was carried out in final volume of 25 µl containing 100 ng DNA, 3 U of Taq DNA polymerase (Chromous Biotech, Bangalore), 2.5 mM $MgCl_2$ (Chromous Biotech, Bangalore), 2.5 mM each dNTPs (Chromous Biotech, Bangalore) and 100 pmol of primers (GeNei, Bangalore). The DNA amplification was performed in the Corbett RG 6000 thermo cycler using the following conditions: complete denaturation (94°C for 5 min), 10 cycles of amplification (94°C for 45 sec, 35°C for 1 min and 72°C for 1.5 min) followed by 30 cycles of amplification (94°C for 45 sec, 38°C for 1 min and 72°C for 1 min) and the final elongation step (72°C for 5 min). All PCR products were separated on 1.5% (w/v) agarose gel and photographed with HP Alpha-imager.

Data Analysis:

The RAPD profiles were analyzed based on the presence or absence of individual RAPD bands. The genetic distance was calculated by the coefficient of similarity of Jaccard. The matrix of genetic distance was used for grouping the lemongrass cultivars based on the Dendrogram constructed by UPGMA (unweighed pair group method with Arithmetic averages).

RESULTS AND DISCUSSION

Medicinal plants have great value in India and many species are known to have medicinal properties which are used in different parts to cure specific ailments since ancient times. In general, *Cymbopogon* plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used for extracting antimicrobial compound for the treatment of infectious diseases. The extraction and isolation of bioactive compounds from plant material is largely dependent on the type of solvent used. In the present investigation, the organic solvent extract of *Cymbopogon* was tested against isolated microorganisms from clinical sample. The extracts of *Cymbopogon* were effective against all microorganisms and their effectiveness varied for different solvent extract. The methanol extract of *Cymbopogon* was highly effective compared to other solvent extract. The minimal inhibitory concentration was determined at different concentrations and varied for different species. Among the tested microorganism *Staphylococcus sp* was most sensitive for methanol extract of all *Cymbopogon* species. The antibacterial activity of the different solvents extract of C*ymbopogon* was determined in isolated pathogenic microorganisms. The observed zone of inhibition was summarized in figure 1, 2 and 3 for *C. martinii, C. citratus and C. nardus* respectively. All the three species of C*ymbopogon* was

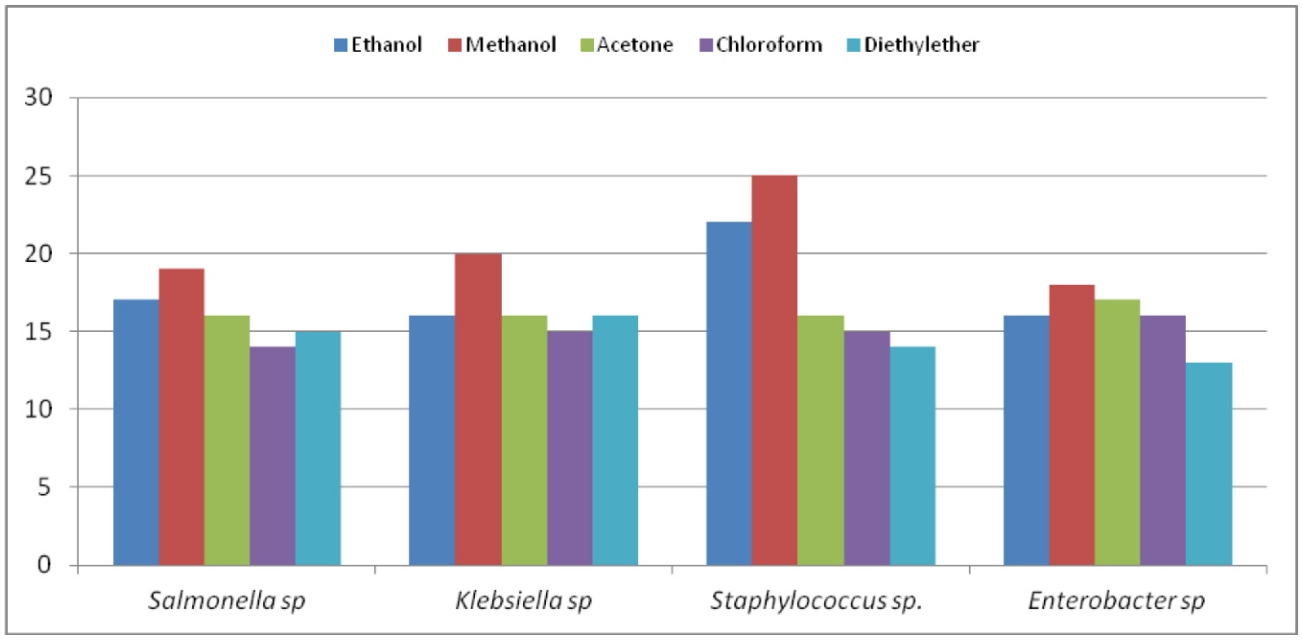

Figure1. Antimicrobial activity of *Cymbopogon citratus*

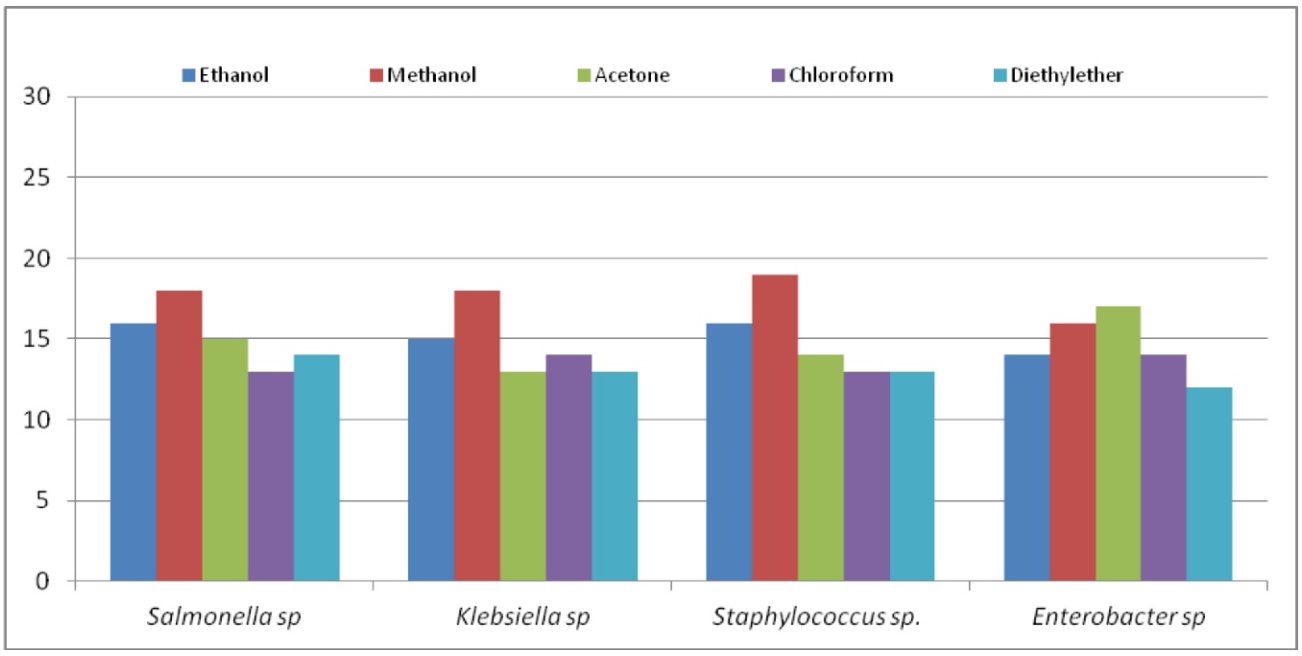

Figure2. Antimicrobial activity of *Cymbopogon martini*

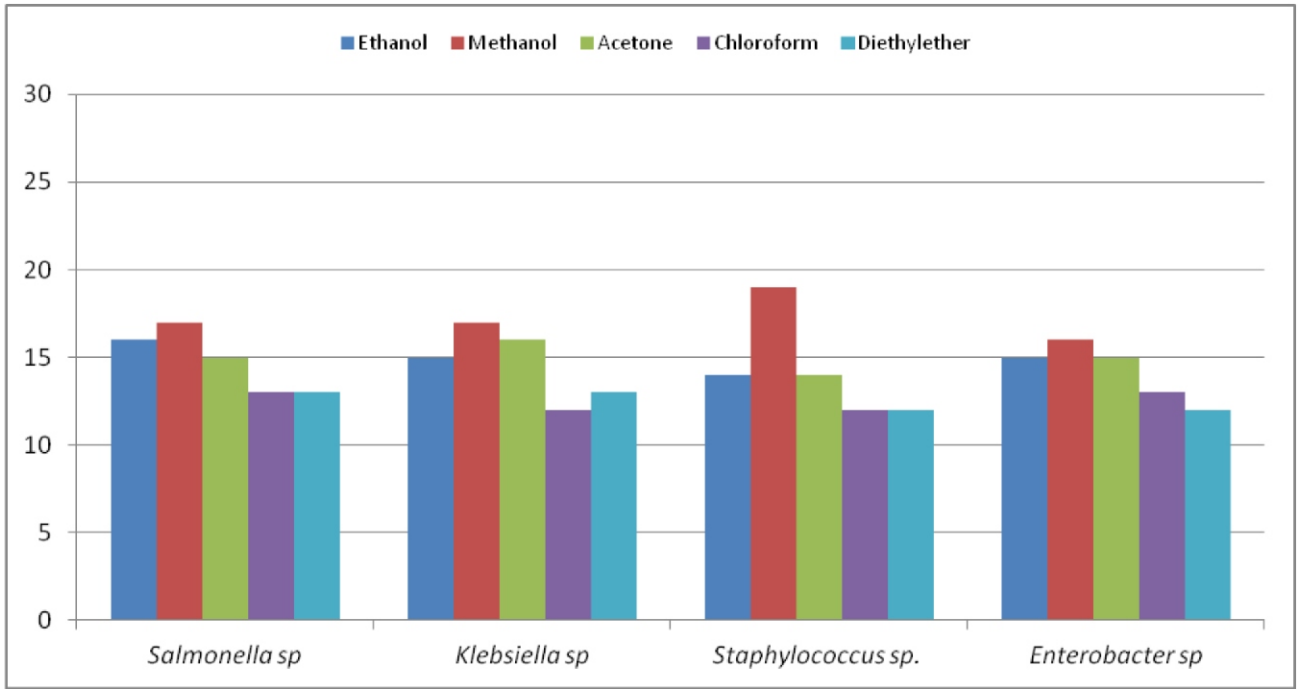

Figure3. Antimicrobial activity of *Cymbopogon nardus*

active against all the tested microorganisms and produced zone of inhibition. The methanol extract produced maximum zone of inhibition and highest (25 mm) zone of inhibition was formed by *C. citratus* against *Staphylococcus sp.* Ethanol and acetone were the other two solvents which produced maximum zone of inhibition.

In the MIC assay all the three species exhibited diverse antimicrobial activity at varied concentrations as summarized in Table 1. The methanol extract was most active compared to other solvent extracts and *Staphylococcus sp.* was most sensitive among tested microorganism.

As the random amplified polymorphic DNA (RAPD) technique uses universal sets of primers, and no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary, it has an advantage over other systems of genetic documentation [17]. Without having prior knowledge of DNA sequences, one can examine the genomic variation by the development of RAPD markers generated by polymerase chain

Table1. Minimal Inhibitory Concentration of *Cymbopogon* Species

Plant Sample and Extraction solvent	Minimum Inhibitory Concentration (Pathogen)			
	Salmonella sp.	*Klebsiella sp.*	*Staphylococcus sp.*	*Enterobacter sp.*
Cymbopogon citratus				
Ethanol	30	30	10	30
Methanol	20	20	10	20
Acetone	30	30	20	20
Chloroform	40	40	30	30
Diethyl Ether	30	30	30	40
Cymbopogon martini				
Ethanol	40	30	30	40
Methanol	30	20	20	30
Acetone	40	40	30	30
Chloroform	40	40	40	40
Diethyl Ether	40	40	40	40
Cymbopogon nardus				
Ethanol	30	30	30	30
Methanol	20	20	10	20
Acetone	30	20	30	30
Chloroform	40	40	40	40
Diethyl Ether	40	40	40	40

reaction (PCR) [18-21]. There are some different methods to form clusters like MCLUST which fits a very similar model to fine structure, K-Means which places individuals in the population closest to them and 'Unweighted Pair Group Method with Arithmetic Mean' (UPGMA) which iteratively merges the closest groups. The aim of a genetic similarity measure is to identify pairs of individuals who are 'closely related' by assigning them higher similarity than those who are distantly related. Similarity matrices can be related to many population genetics methods in a two-stage approach to population genetics by first computing the pair wise similarities, and then perform clustering or other analyses on this summary of the dataset [22, 23]. There are many possible interpretations of what it means for individuals to be closely related, leading to different matrices being constructed. A simple interpretation of relatedness is the average genetic distance [24]. The RAPD patterns of genomic DNA of *C. martinii, C. citratus and C. nardus* by GeNei 1-4 primers (Figure 4) were analyzed for polymorphism. Genetic similarity was calculated using Jaccard's similarity coefficient and dendrogram was generated to access the genetic relationship among three selected species (Figure5). Dendrogram constructed by cluster analysis of RAPD markers showed that *C. martinii* and *C. citratus* are closely related.

CONCLUSION :

Based on the results, it can be concluded that the screening of *Cymbopogon* can make a way for identification of new important antimicrobial components. Further study can be done for determination of toxicity, side effects and pharmaco-kinetic properties of isolated antimicrobial compounds. The genetic diversity presented for the *Cymbopogon species* and variants is valuable for conservation of their germplasm and breeding new varieties of *Cymbopogon*. This molecular analysis of different *Cymbopogon species* by RAPD will generate potential markers for phylogenetic analysis supported by RAPD derived Dendrogram. The Knowledge of the genetic relationships is essential for developing breeding strategies, germplasm management, and utilization of genetic resources [25]. Also

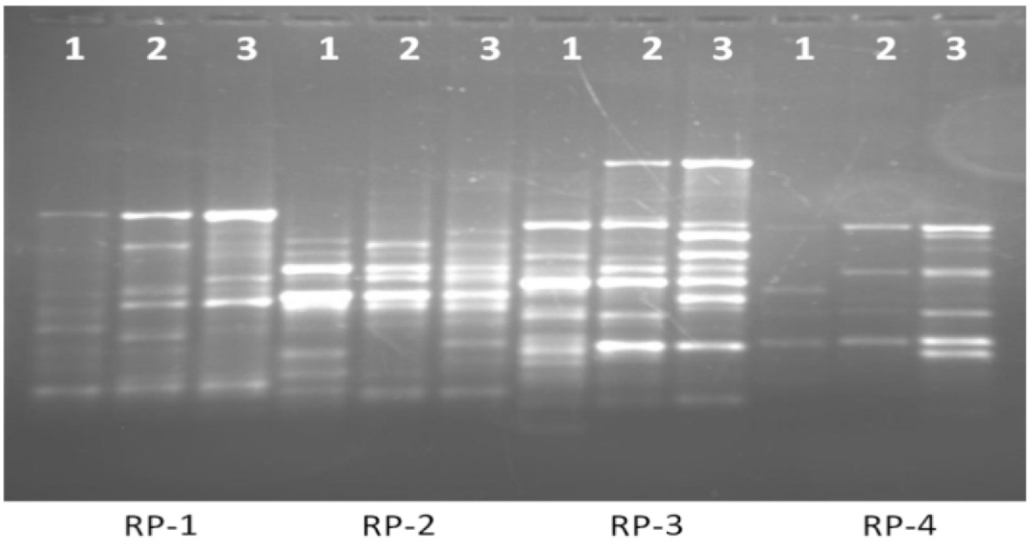

Figure 4. RAPD profiles of three selected cultivars obtained with GeNei (1-4) primers

1-C. *martini,* 2- C. *citratus* and 3- C. *nardus.*

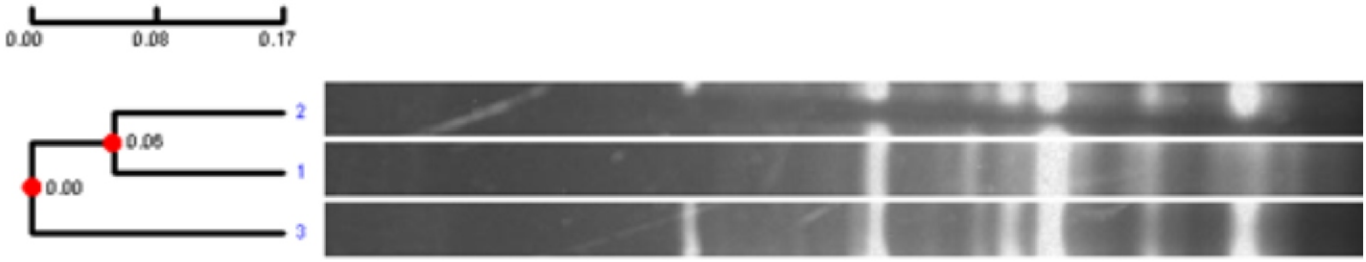

Figure 5. Dendrogram showing genetic relationship among three selected cultivars

1. C. *Citratus,* 2. C. *Martinii,* 3. C. *Nardus*

knowledge of genetic diversity can be used to identify the specific antimicrobial agent in these *Cymbopogon* species. It is important to mention the fact that data results from RAPD assays can be extended to further dissect traits in a more refined way to exactly know the specific genes and genetic pathways using other molecular methodologies. There is also the opportunity and need to study sequences of specific polymorphic bands, to determine the genes detected by RAPD experiments. Further studies with other molecular methodologies are essential to clarify and confirm genetic relationships among plant species depicted using RAPD.

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