ABSTRACT

Ethyl acetate, aqueous, methanol extracts and essential oil of *Cymbopogon citratus* leaves were examined for antifungal activity in *vitro* using the agar well diffusion method, poisoned food technique. Antifungal activity against *Aspergillus flavus* and *Mucor sp* is discussed which were isolated from herbal drugs.

KEYWORDS: *Cymbopogon citratus*, Antifungal, Agar Well Diffusion, Poisoned food technique.

INTRODUCTION

Crude drugs and herbal medicines play an important role in human health care, health improvement, as alternative medicine and materials for medical products in many countries. However, most of them are natural products, and they are shipped after simple washing and then dried without any treatment for sterilization. There is a possibility of microbial contamination from water/soil/animals during production and from human activities such as harvesting, drying and sorting. Trivedi & Singh, 2013 showed the presence of fungi and bacteria in herbal drugs. For the above reasons, the importance of sterilization has been pointed out for quality control of crude drugs and herbal medicines. However, heat treatment is not suitable for them as it may alter the active ingredients/scent of these natural plant/animal derivatives. The quality control of crude drugs has been at the discretion of each pharmaceutical company; therefore, microbial contamination levels vary drastically from company to company. Currently, microbial contamination on crude drugs has become an issue and certain quality assurances have been sought from the Good Manufacturing Practices standpoint. Therefore, it is necessary to estimate the microbial contamination level on crude drugs at each manufacturing stage and to establish contamination assessment methods. Medicinal plants are natural resources, yielding valuable herbal products which are often
used in the treatment of various ailments. *Cymbopogon citratus* is used in detoxifying the digestive organs of the body, like pancreas, liver, kidney and bladder. It stimulates digestion and blood circulation in the body and hence, keeps gastric and indigestion problems at bay (Leite *et al.*, 1986).

**MATERIALS AND METHODS**

**Plant material**

Leaves of *Cymbopogon citratus* were collected from nursery and identified with the help of taxonomic literature, standard flora and herbarium available in the library of Gurukul Kangri University, Haridwar.

**Preparation of plant extract**

Extraction process was done with some modification. Plant materials were washed with water, then surface sterilised and were air dried at room temperature. The samples were ground into a fine powder (Chandrasekaran & Venkatesalu, 2004). The leaves powdered (50g) with distilled water were placed in a soxhlet apparatus for 72 hrs. after which it was filtered with buchner funnel with whatman no. 1 filter paper. The filterate was concentrated using a rotary evaporator to 50ml (Uzama, 2009). The extracts were cooled and stored in a refrigerator at 4°C for analysis. Plant extract was prepared in three solvent i.e aqueous, methanol, and ethyl acetate.

**Extraction of essential oil**

The components of half of the dried leaves were extracted for the essential oil as follows. Two hundred and fifty grams of the powdered leaves was put in a round bottom flask, 1000 ml of distilled water was added and then subjected to hydro distillation in a modified Clevenger apparatus for 8 hours (Bankole, 1997). The oil recovered was dried over anhydrous sodium sulphate and kept in the refrigerator at 4°C before use.

**Source of test fungi and preparation of spore suspension**

Test Fungi *Aspergillus flavus* and *Mucor sp* were isolated from herbal syrup, tonic and crude herbs. The stock suspensions of fungal isolates were standardized to $10^6$ spores/ml by spectrophotometrically at 530 nm and were adjusted to 80 to 85% transmittance. The fungal inoculum ($10^6$ spores/ml) was also determined by plate count on SDA followed by incubation at 25°C±2 for 7 days and observations made for visible growth of fungi at regular interval during the incubation period (Florl *et al.*, 2003; Rasooli and Abyanek, 2004).
Antifungal activity

Agar well diffusion method

The antifungal activity of extracts (aqueous, ethyl acetate and methanolic) of *C. citratus* against *A. flavus* and *Mucor sp.* was evaluated by using agar well diffusion method. SDA plates were inoculated with 100 μl of standardized inoculum (1.5x10^8 CFU/ml) of inoculum (in triplicates) and spread with sterile swabs. Wells or cups of 8 mm size were made with sterile borer into agar plates containing the inoculum and the lower portion was sealed with a little molten agar medium. 300μl volume of the plant extract was poured into a well of inoculated plates. Positive and negative control was also taken. Fluconazole was used as a positive control. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar (Rios, et al., 1988). After incubation for 3-7 days at 25°C, the plates were observed. If antifungal activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the plant extract. The zone of inhibition was measured and expressed in millimeters. Antifungal activity was recorded if the zone of inhibition was greater than 8 mm (Hammer, et al., 1999).

Poisoned food technique

The antifungal activity of plant extracts was evaluated against herbal drug-associated fungi by using poisoned food technique. In poisoned food technique, all the fungi were inoculated on Sabouraud dextrose agar (SDA) plates and incubated for 25°C for 3 to 7 days, to obtain young, actively growing colonies of fungi. Plant extract was mixed with 15ml of cooled (45°C) molten SDA medium and allowed to solidify at room temperature for thirty minutes. A mycelial disc 6 mm diameter, cut out from periphery of 3 to 7 day old cultures, was aseptically inoculated onto the agar plates containing the plant extract. Positive and negative control was also taken. SDA plates with fluconazole were used as positive control. (Georgii and Korting, 1991, McCutcheon et al., 1994)

Statistical Analysis

Statistical analysis of the data was carried out with analysis of variance (one way ANOVA). One-way ANOVA test was performed using “Richard Lowry's web based ANOVA calculator”.

RESULTS AND DISCUSSION

It was found that *Cymbopogon citratus* showing antifungal activity against *Mucor sp* and *Aspergillus flavus*. Ethyl acetate extract showing the highest activity against both pathogen.
Aqueous and methanol extract showing least activity. It was also observed that ethyl acetate extract of *C. citratus* is more active against *Aspergillus flavus* in comparison to *Mucor* (Fig 1, 2). Various researchers have documented the antimicrobial activity of plants including lemongrass, citronella, clove, peppermint, thyme and oregano oils against different fungal species (Mishra and Dubey, 1994; Viudamartos et al., 2007). It was also observed that active extract inhibit the spore germination (fig 1C, 1D, 2C, 2D). It was also observed that agar well diffusion method offer a fast, cost effective and generally accurate result in comparison to poison food technique (Fig 1, 2, 3, 4). So *C. citratus* can be used as both antifungal agent and as preservative in cough syrup and liver tonics.

Fig 1. Antifungal Activity Of *C. Citratus* Against *A. flavus* By Agar Well Diffusion.

A. SHOWING ACTIVITY OF AQUEOUS EXTRACT
B. SHOWING ACTIVITY OF METHANOL EXTRACT
C. SHOWING ACTIVITY OF ETHYL ACETATE EXTRACT
D. SHOWING ACTIVITY OF ESSENTIAL OIL
E. SHOWING ACTIVITY OF ANTIBIOTIC (FLUCONAZOLE)

Fig 2. Antifungal Activity Of *C. Citratus* Against *Mucor* Sp By Agar Well Diffusion Method.

A. SHOWING ACTIVITY OF AQUEOUS EXTRACT
B. SHOWING ACTIVITY OF METHANOL
C. SHOWING ACTIVITY OF ESSENTIAL OIL
D. SHOWING ACTIVITY OF ETHYL ACETATE EXTRACT
E. SHOWING ACTIVITY OF ANTIBIOTIC (FLUCONAZOLE)
Fig 3. Antifungal Activity Of C. Citratus Against A. Flavus By Poisoned Food Technique.
A. SHOWING ACTIVITY OF AQUEOUS EXTRACT
B. SHOWING ACTIVITY OF METHANOL EXTRACT
C. SHOWING ACTIVITY OF ETHYL ACETATE EXTRACT
D. SHOWING ACTIVITY OF ESSENTIAL OIL

Fig 4. Antifungal Activity Of C. Citratus Against Mucor By Poison Food Technique.
A. SHOWING ACTIVITY OF AQUEOUS EXTRACT
B. SHOWING ACTIVITY OF METHANOL EXTRACT
C. SHOWING ACTIVITY OF ESSENTIAL OIL EXTRACT
D. SHOWING ACTIVITY OF ETHYL ACETATE EXTRACT
E. SHOWING ACTIVITY OF ANTIBIOTIC (FLUCONAZOLE)

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Aspergillus flavus</th>
<th>Mucor sp</th>
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<tbody>
<tr>
<td></td>
<td>ZOI(mm)</td>
<td>Std err</td>
</tr>
<tr>
<td>Methanol</td>
<td>-----</td>
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</tr>
<tr>
<td>Ethyl acetate</td>
<td>20±1</td>
<td>1.2</td>
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<tr>
<td>Aqueous</td>
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<tr>
<td>Essential oil</td>
<td>24.3±2.08</td>
<td>0.5</td>
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<tr>
<td>Antibiotic (Fluconazole 5mg/ml)</td>
<td>35.3±0.5</td>
<td>0.3</td>
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* ------ indicates Nill
Values of observed effective zone of inhibition (ZOI), (in mm diameter) excluding the diameter of the well (3 mm) after 2-7 day. incubation against different fungal species when subjected to different extracts in agar well diffusion assay. Assay was performed in triplicate and results are the mean of three values ±Standard deviation. In each well, the sample size was 100 μl. HSD= the absolute [unsigned] difference between any two sample means required for significance at the designated level. HSD[.05] for the .05 level; HSD[.01] for the .01 level. M1= mean of ethyl acetate plant extract, M2= mean of plant essential oil and M3= mean of antibiotic, Std err- standard error

Table 2. Showing Antifungal Activity Of C. Citratus Against A. Flavus & Mucor By Poison Food Technique

<table>
<thead>
<tr>
<th>PLANT EXTRACT</th>
<th>Aspergillus flavus</th>
<th>Mucor sp</th>
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<tbody>
<tr>
<td>Aqueous</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Ethyl acetate</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Methanol</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Essential oil</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Antibiotic (Fluconazole 5mg/ml)</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* - no inhibition, ++ good inhibition, +++ best inhibition

FIG 5. Comparative analysis of different extracts of Cymbopogon citratus against fungal isolates.
CONCLUSION

The microbial contamination of herbal drugs is a major reason of decline of their demand in global herbal market. Singh et al, 2012 isolated actinomycetes from ayurvedic drugs. The growing concern about drug safety has recently led to the development of natural antimicrobials to control spoilage microorganisms. Herbs are one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavour (Nevas et al., 2004, Souza et al., 2005). Finding of present study show that *C.citratus* exhibited antifungal activity and hence *C.citratus* can be used as an antifungal agent in syrups and tonic. Plant extract have a role as preservatives. This study indicated that plant extract and essential oils may possess antifungal activity and can be exploited as an ideal treatment for future herbal drug contaminant for eliminating fungal spread. Recently, there has been a considerable interest in extracts and essential oils from aromatic plants with antimicrobial activities for controlling pathogens and/or toxin producing microorganisms in foods (Reddy et al., 1998; Soliman & Badeaa, 2002; Valero & Salmeron, 2003).

REFERENCES


