Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics,
Pharmaceutical and Chemical Sciences

Journal Home page <http://www.rjlbpcs.com/>

Original Research Article

DOI - 10.26479/2017.0304.03

ANTIFUNGAL ACTIVITY OF *Cymbopogon nardus* ESSENTIAL OIL AGAINST SOME FUNGI CONTAMINATING CEREALS AND PULSES

H. J. KRO¹, S. DAS² and K. TAYUNG^{1*}

1. Mycology and Plant Pathology Laboratory, Department of Botany, Gauhati University, Guwahati Assam, IN

2. Department of Botany, North Orissa University, Takatpur, Baripada- 757003, Odisha, India

ABSTRACT: In the present investigation antifungal activity of *Cymbopogon nardus* essential oil was studied against some fungi contaminating cereals and pulses. A total of 172 fungal isolates were obtained from contaminated cereals and pulses samples collected from Jalukbari and Maligaon markets of West Guwahati, Assam. The frequently isolated fungal species were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and fungi belonging to genera *Penicillium* and *Mucor*. Essential oil of *Cymbopogon nardus* was obtained by hydro-distillation using Clevenger apparatus. Antifungal efficacy of the essential oil at different concentration against the isolated fungi was carried out by Agar Cup Diffusion assay. The result indicated all the fungal species showed varying degree of antifungal activity. However, four fungal species showed considerable zone of inhibition. Amongst them at 12.5% oil concentration *Aspergillus niger* was most susceptible with a zone of inhibition of 41mm followed by *Aspergillus flavus* (21mm) and *Aspergillus fumigatus* (17mm). One of the *Penicillium* species also showed promising activity showing 16mm inhibition zone. The bioactive components of *Cymbopogon nardus* essential oil was studied by GCMS analysis. The result showed one major spectrum with four medium and five minor spectra indicating the presence of bioactive metabolites. Since most of the isolated fungal genera produced aflatoxin which causes toxicity to living organisms including humans, the inhibition of these fungi genera by the essential oil of *Cymbopogon nardus* is promising eco-friendly approach to control such contamination.

KEYWORDS: *Cymbopogon nardus*, Essential oils, Antifungal activity, GCMS analysis

***Corresponding Author: Dr. Kumanand Tayung Ph.D.**

Mycology and Plant Pathology Laboratory, Department of Botany, Gauhati University, Guwahati- 781014, Assam, India. * Email Address: kumanandbotnou@rediffmail.com

1. INTRODUCTION

Food products mostly cereals and pulses are important nutrient source worldwide and their chemical composition attract both bacterial and fungal colonizers particularly moulds. After successful colonization of the product, its nutritional properties are altered which can be accompanied by the production of toxic secondary metabolites which may result in grave medical problems, and is an issue that needs continuous awareness with respect to food safety. Storage fungi infect the grain during storage in improper conditions like excessive humidity. Most of the post harvest contaminating filamentous fungi produces mycotoxins as their secondary metabolite (Samson *et al.*, 2004 and Pitt *et al.*, 2009). Mycotoxins are well known carcinogens, mutagens, nephrotoxicants and neurotoxicants to a wide range of organisms (Refai, 1998). Use of synthetic chemicals to control growth of fungal contaminant could have serious health consequences as most of the chemicals have negative effects to living cells and the environments. Therefore, the uses of various natural eco-friendly alternatives such as essential oils to control mycotoxins producing fungi have been given priority in the recent years. Aromatic and medicinal plant essential oils and their components demonstrate antibacterial, antifungal, and food preservative activities against a wide range of microbial pathogens (Basim *et al.*, 2000; Tripathi and Kumar, 2007; Pandey *et al.*, 2014b; Sonker *et al.*, 2015; Gormez *et al.*, 2016). These essential oils are hydrophobic liquids of aromatic compounds that are volatile and oily in nature and present in various plant parts such as twig, flower, leaf, bark, seed, and root. Many plant essential oils are useful as a flavor or aroma enhancer in cosmetics, food additives, soaps, plastics resins, and perfumes. Moreover, curiosity about essential oil applications that can act as antimicrobial agents is growing because of the broad range of activities, natural origins, and generally recognized as safe (GRAS) status of essential oils. *Cymbopogon nardus* is an aromatic, evergreen, clump-forming perennial grass belonging to the family Poaceae. Their essential oil are extracted from the aerial parts of the plant and is known as citronella oil, and has been traditionally used as mosquito repellent, household fumigant, or fragrance agent in food commodities, soaps and cosmetics (Chomchalow, 1993; Corner and Watanabe 1969; Jantan and Zaki, 1999). Many studies have reported on the antifungal and antimicrobial property of *C. nardus* essential oil. Billerbeck *et al.*, (2001) claimed that essential oil of *C. nardus* at concentration of 400 mg/L could inhibit 80% of *Aspergillus niger* growth. Meanwhile, Oussalah *et al.*, (2006) reported that the essential oil showed antimicrobial activity at concentration of 4 mg/mL against *Pseudomonas putida* CRDAV 372 isolated from fresh beef. Nakahara *et al.*, (2003) studied the chemical composition of citronella oil and its antifungal activity. The crude essential oil markedly suppressed the growth of several species of *Aspergillus*, *Penicillium* and *Eurotium*. Therefore the present work was carried out to evaluate the efficacy of *Cymbopogon nardus* essential oil extracted from leaf by hydro-distillation in controlling fungi contaminating post harvested stored grains. An attempt has been made to characterize the various components of the essential oil by GCMS analysis.

2. MATERIALS AND METHODS

Collection and isolation of fungi from post harvested stored grains

Stored food grains of two cereals viz., *Triticum aestivum* and *Oryza sativa* and two pulses viz., *Lens culinaris* and *Arachis hypogaea* were collected from the nearby market of Jalukbari and Maligaon of West Guwahati, Assam, India. For isolation of associated fungi from the collected samples two methods were used (serial dilution and direct plating). Ten (10) grains of each sample was added to 10ml of sterilized distilled water and shaken for 3 minutes to get a stock solution. 1ml of the stock was pipetted into 9ml of sterilized distilled water in a test tube to make a serial dilution of 10^{-1} , 1ml of 10^{-1} serial dilution was again pipetted into 9ml of sterilized distilled water in a test tube gave 10^{-2} serial dilution. Similar method was carried out to give final concentrations of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} dilution. Each dilution was inoculated into Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA) media. While in the direct isolation, ten (10) contaminated grains of each sample were directly inoculated in Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA) media. Inoculated samples in the media were incubated at $30^{\circ}\pm 2^{\circ}\text{C}$ for 48-72 hours. Fungi growing out of the inoculated samples were carefully isolated as pure culture as stored in 4°C in refrigerator for further study. The isolated fungal species were identified based on their colonial traits and detailed microscopic studies using standard identification manual of Gilman (1971).

Extraction of essential oil from leaves of *Cymbopogon nardus*

Essential oil (EO) was extracted from the fresh leaves of *Cymbopogon nardus* by Clevenger's hydro-distillation apparatus. EO so obtained was collected in polypropylene vials and sealed with parafilm to prevent vaporization of EO. It was then stored in refrigerator at 4°C for use in further assay.

Determination of antifungal activity of the essential oil

The antifungal activity of the essential oil against the tested fungal species was performed by Agar cup diffusion method. For this purpose, first the culture of the tested fungal species were inoculated in Potato Dextrose Broth and incubated at 30°C for 5-6 days till sporulation. At the meantime Potato Dextrose Agar plates were prepared and 1ml of the broth culture of sporulating test fungi were inoculated on the respective PDA plates and it was evenly inoculated throughout the PDA plate with the help of sterilized cotton swabs. Now agar cups were prepared by scooping out the medium with the help of sterile cork borer (7mm in diameter). Each cup was then loaded with different concentration of essential oils separately. The plates were incubated at 30°C for 48-72 hours and the zone of inhibition was measured thereafter.

Characterization of essential oil of *Cymbopogon nardus*

The essential oil was characterized and identified by GCMS analysis. Chromatography was performed on a DB-Wax capillary column (60 m $\times$ 0.25 mm ID and 0.25 μm film thickness). The electron impact technique (5.0 mV) was used. The carrier gas was helium at a flow rate 1.5625pts/s sample was injected. The injector and detector temperatures were 350°C and 260°C , respectively.

The column oven was programmed as follows: initial temperature 50°C; initial time 50min; final temperature 350°C; final time 50min. The sample was dissolved in methanol and a split injection technique was used. The identification of the compounds was based on comparison of their retention indexes (RI) and retention time. They were also confirmed by comparison of their mass spectra with the NIST/NBS-Wiley library spectra and literature data.

3. RESULTS AND DISCUSSION

A total of 172 fungal isolates were isolated from contaminated cereals and pulses samples collected from Jalukbari and Maligaon markets out of which 44 isolates were obtained from *Oryza sativa*, 47 isolates from *Triticum aestivum*, 42 isolates from *Arachis hypogaea* and 39 isolates from *Lens culinaris*. The frequently isolated fungal species were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and fungi belonging to genera *Penicillium* and *Mucor* (Table-1 and Table-2).

Table-1: Occurrence of fungal species isolated from contaminated cereals

Fungi	No. of isolates	
	<i>Oryza sativa</i>	<i>Triticum aestivum</i>
<i>Aspergillus niger</i>	09	04
<i>Aspergillus fumigatus</i>	05	12
<i>Aspergillus flavus</i>	14	16
<i>Penicillium nordicum</i>	03	04
<i>Penicillium italicum</i>	10	07
<i>Mucor</i> sp.	03	04
Total	44	47

Table-2: Occurrence of fungal species isolated from contaminated pulses

Fungi	No. of isolates	
	<i>Arachis hypogaea</i>	<i>Lens culinaris</i>
<i>Aspergillus niger</i>	12	05
<i>Aspergillus fumigatus</i>	04	11
<i>Aspergillus flavus</i>	16	13
<i>Penicillium nordicum</i>	05	03
<i>Penicillium italicum</i>	03	04
<i>Mucor</i> sp.	02	03
Total	42	39

Determination of antifungal activity of oil against tested fungal species

The essential oil extracted from the fresh leaves of *Cymbopogon nardus* through Clevenger's hydro-distillation apparatus showed varying degree of inhibition against the test fungal isolates at different oil concentration (Table-3). At 50% concentration it showed highest activity against all the isolates where no visible fungal growth was observed. At 25% concentration it showed highest activity against *Aspergillus niger* showing 50mm zone of inhibition. It was followed by *Aspergillus flavus*, *Aspergillus fumigatus* and *Penicillium italicum* with 39mm, 28mm and 27mm zone of inhibition respectively. At 12.5% concentration it showed highest activity against *Aspergillus niger* with 41mm zone of inhibition. It was followed by *Aspergillus flavus*, *Aspergillus fumigatus* and *Penicillium italicum* with 21mm, 17mm and 16mm zone of inhibition respectively.

Table-3: Antifungal activity of the essential oils of *Cymbopogon nardus* at different oil concentration:

Zone of inhibition (mm)			
Fungal species	50% of EOs	25% of EOs	12.5% of EOs
<i>Aspergillus niger</i>	90	50	41
<i>Penicillium italicum</i>	90	27	16
<i>Aspergillus fumigatus</i>	90	28	17
<i>Aspergillus flavus</i>	90	39	21

* Different concentration of essential oil was prepared by dissolving the oil with DMSO

Identification of the essential oil by GCMS analysis

To find out the bioactive compounds that may be responsible for the antifungal activity, the essential oil of *Cymbopogon nardus* was characterized by GCMS analysis. The result showed a major spectrum with four medium and five minor spectra indicating presence of bioactive compounds. The identification of the compounds was based on comparison of their retention indexes (RI) and retention time. They were also confirmed by comparison of their mass spectra with the NIST/NBS-Wiley library spectra and literature data (Table-4). 2-methyl-undecanal was identified as the major compound.

Figure- 4: Chromatogram of essential oil of *Cybopogon nardus* by GCMS analysis**Table-4: Identified bioactive compounds of *Cymbopogon nardus***

Sl. No.	Peak Id	Name of the Compound	MW
01	12.805	Camphene	136
02	15.301	D-limonene	136
03	19.088	3,7-dimethyl-6-octenal	154
04	21.274	3,7-dimethyl-6-octen-1-ol	156
05	21.694	(z)-3,7-dimethyl-2,6-octadienal	152
06	22.029	(e)-3,7-dimethyl-2,6-octadien-1-ol	154
07	22.519	3,7-dimethyl-2,6-octadienal	152
08	24.495	3,7-dimethyl-6-octen-1-olpropanoate	212
09	25.270	(z)- 3,7-dimethyl- 2,6-octadien-1-ol acetate	196
10	26.741	Caryophyllene	204

Post-harvest deterioration of food grains and other food products are one of the major reasons for severe crop production losses in the world. India is a world leader in the production of food grains and protection of grain in storage in bags or bulk is important to assure food security. Losses of stored grain worldwide are in the range of 5-10% or about 20 million tons a year with insects and molds, and can exceed to more than 50% if one has to include losses due to rodents and birds. Numbers of factors together contribute for grain damage and deterioration and these include agro-climatic factors, socio economic conditions, quality of seeds sown and time of harvest and methods of processing the food grains. Fungal colonization on grains and food products results in discolorations, decreased germination and vigor, heating, mustiness, dry matter loss of the grain and finally deterioration in nutritional quality. As the mould grows on food it produces enzymes that break down the food resulting in spoilage. In addition to enzymes, some moulds produce mycotoxins on the food. Ingestion of mycotoxin contaminated food is fatal. Hundreds of people in developing countries die every year after consuming grains contaminated with mycotoxins. In the present investigation post harvested stored contaminated grains were studied for associated fungi species. The samples were collected from local stored market of West Guwahati, Assam. The most frequently isolated fungal species were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and species of *Penicillium*. In many instances these fungal genera were commonly isolated from contaminated stored grains (Logrieco and Visconti, 2004; Kocic-Tanackov and Dimic, 2013). Further, Prakash *et al.*, (2015) have also reported major fungi found associated with stored food items include *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., *Curvularia* spp., members of Mucorales etc. Storage fungi are generally present as mycelia below the pericarp, or as dormant spores on the surface of seeds. They cause spoilage of stored foods through discoloration, loss of viability, heating and mustiness, biochemical changes leading to quality loss and production of toxins. Among the most serious is *Aspergillus flavus*, which produces 'aflatoxin' on many grains and oilseeds, and causes quality deterioration. Aflatoxin and other mycotoxins are highly poisonous and carcinogenic compounds. *Aspergillus* and *Penicillium* are important fungi that are generally associated with stored products. The nutritional status of grain, moisture, temperature, infestation by insects, mites and foreign matter, influence the microbial invasion and subsequent spoilage during storage. A number of storage fungi attacked stored foodstuffs and caused some loss to the grains. However, the most important damage is caused by *Aspergillus flavus*, which produces aflatoxin, a substance toxic to animal including man, as it has a strong positive association with the risk of developing primary liver cancer. In one of the farm surveys Quitco *et al.*, (1987), reported that groundnuts in storage at farms, aflatoxin continued to increase at the rate of 1.4 ppb per day, while in the wholesalers' warehouse for more than three months contained 275 ppb aflatoxin. In the present study also *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* were isolated as contaminant from stored post harvested grains. These fungi were normally reported to produce mycotoxins (Begum and Samajpati, 2000). In many low-

income countries, mycotoxins, and particularly aflatoxin, affect staple foods including cereals (maize, wheat and rice principally) and their derivatives; oilseeds (cotton, peanut, rapeseed, coconut, sunflowers and others), cassava, groundnuts and other nuts, and a great variety of foods which are consumed by humans like dry fruits, delicatessen products, spices, wines, legumes, fruits, milk and milk derivatives. The problem of aflatoxin contamination of the food products is a common problem in tropical and subtropical regions of the world especially in the developing countries such as the sub-Saharan countries with poor practices and where the environmental conditions of warm temperatures and humidity favors the growth fungi (Bennett and Klich, 2003). The various food products contaminated with aflatoxin include cereals like maize, sorghum, pearl millet, rice and wheat; oilseeds such as groundnut, soybean, sunflower and cotton; spices like chilies, black pepper, coriander, turmeric and zinger; tree nuts such as almonds, pistachio, walnuts and coconut; and milk and milk products (Lopez *et al.*, 2002). Maize and groundnuts are major sources of human exposure because of their greater susceptibility to contamination and frequent consumption throughout the world. In the present investigation several mycotoxins producing fungal species namely *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus* and *Penicillium* species were isolated from ground nut (*Arachis hypogaea*). The result corroborate with the finding of several workers (Sinha and Sinha, 1991). The mycoflora of stored wheat grains predominantly consisted of ubiquitous mould genera *Aspergillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Mucor*, *Rhizopus* and *Penicillium* possibly because of their omnipresence, capacity to grow on all possible substrates and a wide range of temperature and humidity. The grain losses found in quantity and quality; can be in the form of depletion in seed viability, hardness, colour, size and shape, grain weight and various biochemical parameters viz., protein, carbohydrate and vitamins under post harvest storages. In the present study, occurrence of *Aspergillus* species was found in higher stored grains indicating the resemblance with results of previous workers (Magan, *et al.*, 2003; Mathew *et al.*, 2010). Amongst the mycoflora associated with stored grains, *Aspergillus* is known to produce mycotoxins that deteriorate the quality of stored grains; it becomes quite essential to protect the stored grains from fungal infection by undertaking necessary steps to prevent qualitative and quantitative losses of stored grains.

Natural plant compounds have been used traditionally to preserve foods in countries like Japan, India and Russia (García-Cela *et al.*, 2012). In the present study antifungal activity of the fungal contaminant isolated stored grains was evaluated by using essential oil. Botanicals are plants or plant-derived products having active ingredients for the control of storage pests. These are (i) spices, and (ii) medicinal and other plants. In addition to being used to flavour foods, spices have been used from ancient times to protect stored products from pests. Traditionally, pieces of dried spices or ground spices were used to sprinkle over or mix with stored foods, but recently the use of extracts or oils has been experimentally tried with encouraging results. The results of our present study also revealed that essential oil could be successfully used for inhibiting many of the aflatoxins

producing fungi isolated from contaminated grains. Essential oils have played an important role in stored food protection, as they are effective, easily accessible and applicable (normally by mixing with the stored products). Essential oils contain mainly terpenoids, including monoterpenes, sesquiterpenes, and other terpene derivatives. Most monoterpenes are pleasantly aromatic and exhibit low toxicity to mammals, making them good candidates for use as insecticides for pest control in stored grains and other stored food products. Several essential oils have been successfully applied to control and inhibit aflatoxins producing fungi. Similar result was also obtained in our present study, where essential oils extracted from *Cymbopogon nardus* significantly inhibited fungi species producing aflatoxins. Palevitch and Craker (1994) in their review article had emphasized the development of fungicides based on essential oils in the control of storage fungi. Many workers have found that a number of essential oils such as: eucalyptus, lavender, lemongrass, rosemary, bergamot, cinnamon leaf, wormwood, turpentine, etc., possess potent antibacterial and antifungal properties. EOs are composed of a number of different components such as terpenes, aromatic phenols, oxides, ethers, alcohols, esters, aldehydes and ketones in different composition or combinations. Some of the components remain present in very high concentration while some in very low concentration. Such finding collaborates with our recent analysis of essential oils by GCMS in which major component were found to be present. The antifungal efficacy of EO is mainly either attributed to the overall synergistic effects of all the major and minor compounds or to the bioactivity of the major compounds (Mishra *et al.*, 2013). The chemical composition can vary according to method of EO isolation, age of plant, time of harvest, ecological and geographical variations. Hence, before large scale application the chemical standardization of EO must be endorsed. Since most of the isolated fungal genera known to produce aflatoxin which causes toxicity to living organisms including humans, the inhibition of these fungi genera by the essential oil of *Cymbopogon nardus* is promising eco-friendly approach to control such contamination.

ACKNOWLEDGEMENT

We are thankful to head of the Department of Botany, Gauhati University from providing all the necessary facilities required for the investigation. We also acknowledge IIT-G biotech park for helping in the GCMS analysis.

CONFLICTS OF INTEREST

Author has no conflicts interest

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