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Fungi Associated with Contaminated Stored Grains and their Biological Control using *Zanthoxylum rhetsa* Essential Oil

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Abstract: In the present investigation antifungal activity of essential oil obtained from *Zanthoxylum rhetsa* was studied against some fungi contaminating stored grains. A total of 83 fungal isolates were obtained from contaminated samples collected from West Guwahati markets, India. The frequently isolated fungal species were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and fungi belonging to genera *Penicillium* and *Mucor*. Essential oil of *Zanthoxylum rhetsa* was obtained by hydro-distillation using Clevenger apparatus. Antifungal efficacy of the essential oil against the isolated fungi was carried out by Agar Cup Diffusion assay. The result indicated that all the fungal species showed varying degree of antifungal activity. Amongst the fungi, *Aspergillus niger* showed high susceptibility toward the oil followed by *Aspergillus fumigatus* and *Penicillium italicum* while least antifungal activity was observed in *Aspergillus flavus*. The essential oil of *Zanthoxylum rhetsa* was characterized by GCMS analysis. The result indicated one strong peak and two minor peaks 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 *Zanthoxylum rhetsa* is promising eco-friendly approach to control such contamination.

Keywords: *Zanthoxylum rhetsa*, Essential oils, Antifungal activity, GC-MS analysis

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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 that may result into grave medical problems. This is an issue that needs our continual 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; 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. *Zanthoxylum rhetsa* is a shrubs belonging to the family Rutaceae. The plant has several ethnomedicinal uses. Tribal communities of North East India use the leaf of the plant as vegetables. The leaf of the plant has a pungent smell due to presence of oil glands. Therefore the present work was carried out to evaluate the efficacy of *Zanthoxylum rhetsa* essential oil at different concentration in controlling fungi contaminating post harvested stored grains. An attempt was also made to characterize the compounds present in the oil by GCMS analysis.

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 West Guwahati markets, 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 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) media. While in the direct isolation, ten (10) contaminated grains of each sample were directly inoculated in Potato Dextrose Agar (PDA) media. Samples were inoculated in the media and incubated at 30°±2°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 *Zanthoxylum rhetsa*

Essential oil (EO) was extracted from the fresh leaves of *Zanthoxylum rhetsa* through Clevenger's hydro-distillation apparatus. EO so obtained was collected in polypropylene vials and sealed their cap 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 *Zanthoxylum rhetsa*

The essential oil was characterized and identified by GCMS analysis. Chromatography was performed on a DB-Wax capillary column (60 m × 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.

Results

A total of 83 fungal isolates were isolated from contaminated cereals and pulses samples collected from West Guwahati market, India. The frequently isolated fungal species were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and fungi belonging to genera *Penicillium* and *Mucor* (Table 1).

Table 1: Occurrence of fungal species isolated from contaminated food grains

Fungi	No of isolates
Aspergillus niger	21
Aspergillus fumigatus	16
Aspergillus flavus	20
Penicillium nordicum	07

Penicillium italicum	14
Mucor sp.	05
Total	**83**

Determination of antifungal activity of oil against tested fungal species

The essential oils extracted from the fresh leaves of *Zanthoxylum rhetsa* through Clevenger's hydro-distillation apparatus showed varying degree of inhibition against the test fungal isolates at different oil concentration (Table 2). At 50% concentration it showed highest activity against *Aspergillus niger* with 47mm zone of inhibition. It was followed by *Aspergillus fumigatus*, *Penicillium italicum* and *Aspergillus flavus* with 40mm, 35mm and 33mm zone of inhibition respectively. At 25% concentration it showed highest activity against *Aspergillus niger* with 42mm zone of inhibition. It was followed by *Aspergillus fumigatus*, *Penicillium italicum* and *Aspergillus flavus* with 28mm, 27mm and 25mm zone of inhibition respectively. At 12.5% concentration it showed highest activity against *Aspergillus niger* with 30mm zone of inhibition. It was followed by *Aspergillus fumigatus*, *Aspergillus flavus* and *Penicillium italicum* with 24mm, 19mm and 18mm zone of inhibition respectively.

Table 2: Antifungal activity of essential oil of *Zanthoxylum rhetsa* at different oil concentration

Zone of inhibition (mm)			
Fungal species	***50% of EO**	**25% of EO**	**12.5% of EO**
Aspergillus niger	47	42	30
Penicillium italicum	35	27	18
Aspergillus fumigatus	40	28	24
Aspergillus flavus	33	25	19

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

Identification of bioactive compounds of essential oils by GCMS analysis

To find out the bioactive compounds that may be responsible for the antifungal activity present in the essential oils of these two plant samples, GCMS analysis was carried out. The result indicated one strong signal and two minor peaks in the essential oil of *Zanthoxylum rhetsa* indicating the presence of bioactive metabolites (Figure 1). 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 3). 2-methyl-undecanal was identified as the major compound.

Table 3: Identified bioactive compounds of *Zanthoxylum rhetsa*

Sl. No.	Peak ID	Name of the Compound	MW
01	12.250	3-carene	136
02	17.182	2-methyl-undecanal	184
03	23.134	2-pentacosanone	366

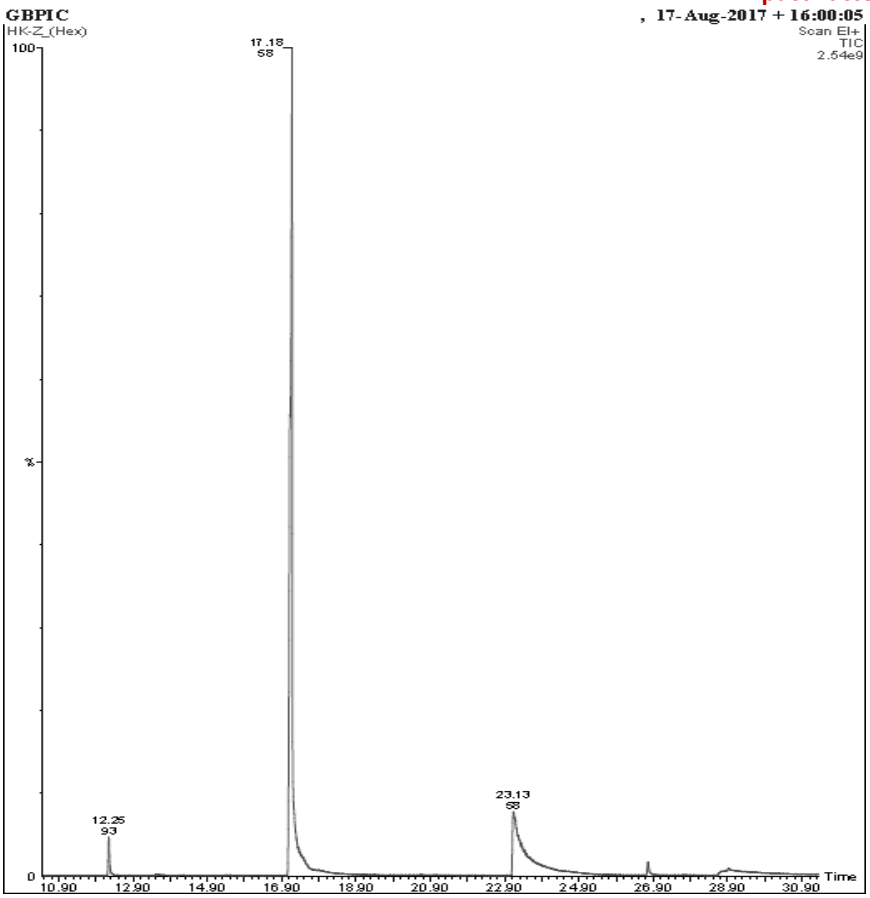

Figure 1: Chromatogram of essential oil of *Zanthoxylum rhetsa* obtained by GCMS analysis

Discussion

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 e*t 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 derivates; 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 derivates. 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 spinkle 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). The essential oils effective against stored insects are those which contain 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 *Zenthoxylum rhetsa* 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.

Conclusion

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. As the mould grows on food produces enzymes that break down the food resulting in spoilage. In addition to enzymes, some moulds produce mycotoxins on the food and ingestion of mycotoxin contaminated food is fatal. 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 *Zanthoxylum rhetsa* is promising eco-friendly approach to control such contamination.

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Supplementary figures

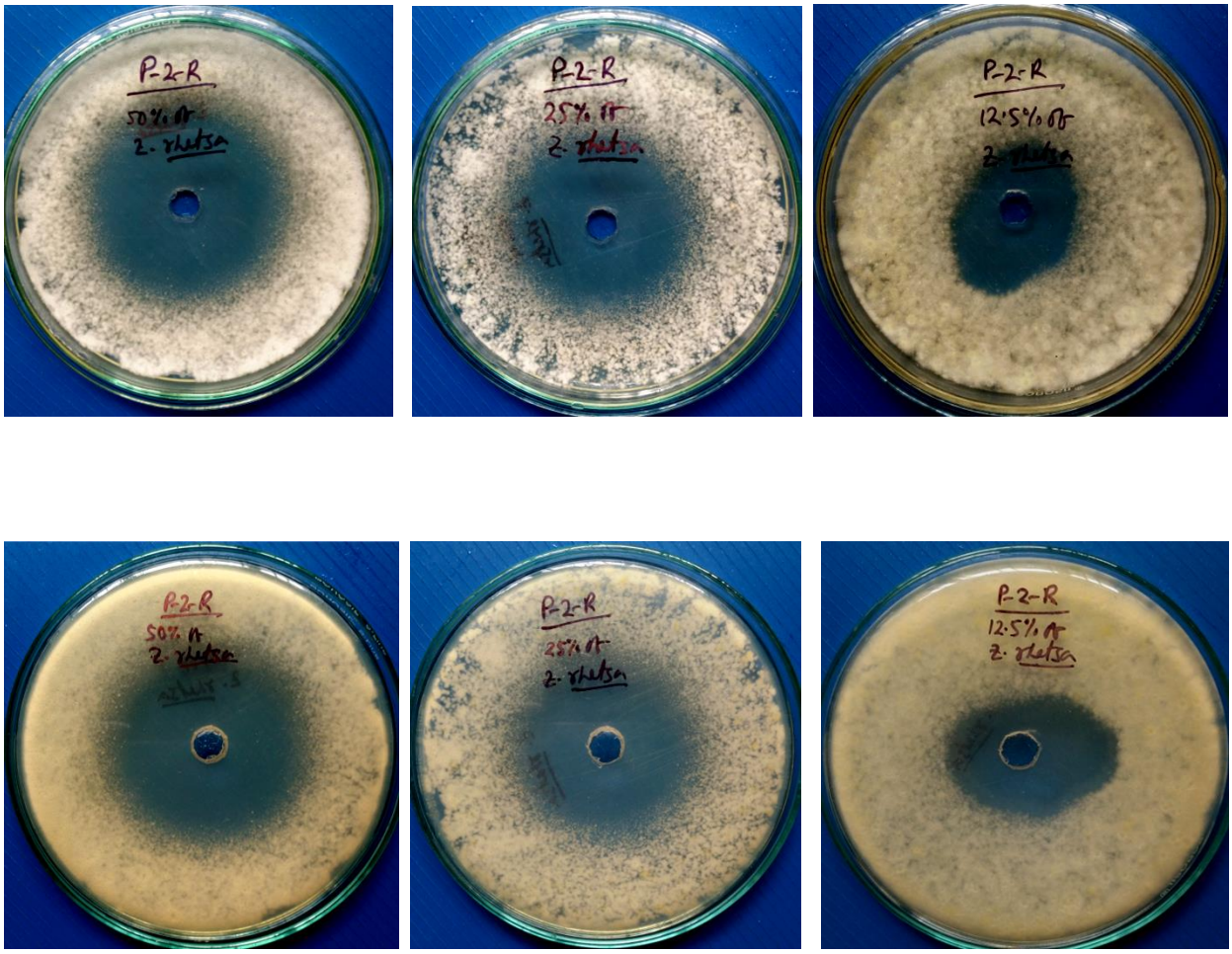

Figure 2: Antifungal activity of the essential oils of *Zenthoxylum rhetsa* at different oil concentration against *Aspergillus niger*.

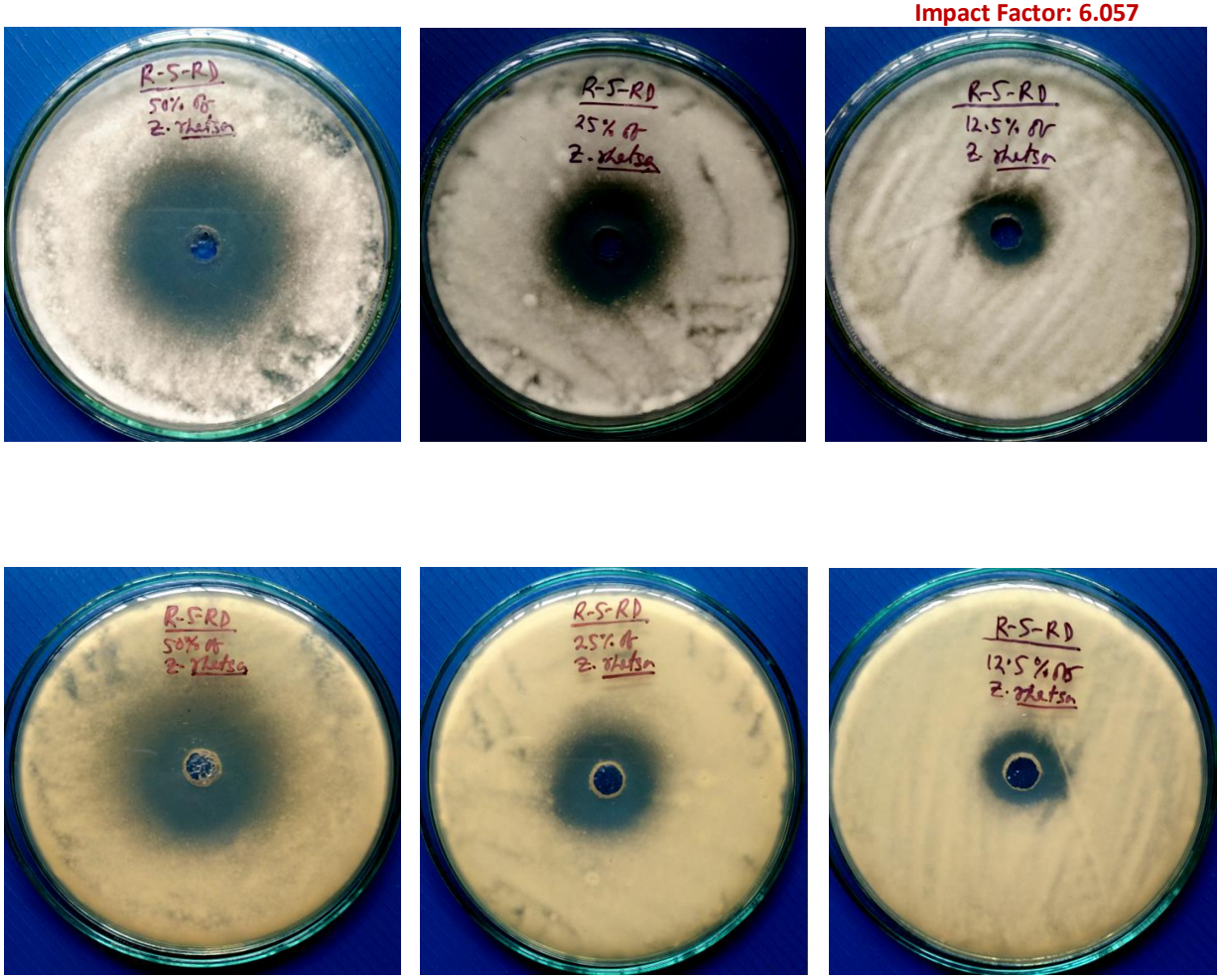

Figure 3: Antifungal activity of the essential oils of *Zenthoxylum rhetsa* at different oil concentration against *Aspergillus flavus*.

