

**SCREENING OF ESSENTIAL OILS, PLANT EXTRACTS AND
SYNTHETIC CHEMICALS FOR THE MANAGEMENT OF
Aspergillus flavus AND *Rhizoctonia bataticola***

UDAY KUMAR T. U.

**DEPARTMENT OF MOLECULAR BIOLOGY AND
AGRICULTURAL BIOTECHNOLOGY
COLLEGE OF AGRICULTURE, RAICHUR
UNIVERSITY OF AGRICULTURAL SCIENCES**

RAICHUR – 584 104

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Aspergillus flavus AND *Rhizoctonia bataticola***

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By

UDAY KUMAR T. U.

**DEPARTMENT OF MOLECULAR BIOLOGY AND
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CERTIFICATE

This is to certify that the thesis entitled “**SCREENING OF ESSENTIAL OILS, PLANT EXTRACTS AND SYNTHETIC CHEMICALS FOR THE MANAGEMENT OF *Aspergillus flavus* AND *Rhizoctonia bataticola***” submitted by **Mr. UDAY KUMAR T. U.** for the award of degree of **MASTER OF SCIENCE (AGRICULTURE)** in **MOLECULAR BIOLOGY AND AGRICULTURAL BIOTECHNOLOGY** to the University of Agricultural Sciences, Raichur, is a record of bonafide research work done by him during the period of his study in this University, under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

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Date: JULY, 2018

(**KISAN. B.)**

Approved by

Chairman:

(**KISAN. B.)**

Members: 1. _____

(**AYYANGOUDA PATIL**)

2. _____

(**I. SHANKERGOUD**)

3. _____

(**Y. S. AMARESH**)

4. _____

(**B. V. TEMBHURNE**)

Affectionately Dedicated

To

My Beloved PARENTS

Smt. Chandramma. S.

Shri. Umamaheshwarappa. T. S.,

SISTERS

AND

FARMING COMMUNITY

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LIST OF ABBREVIATIONS

/	:	Per
%	:	Per cent
+	:	Plus
=	:	Equals
>	:	Greater than
°C	:	Degree Celsius
µg	:	Microgram
µl	:	Microliter
µM	:	Micromole
APEDA	:	Agriculture and Processed Food Products Export Development Authority
CAT	:	Catalase
CD	:	Critical Difference
cm	:	Centimeter
CoA	:	Coenzyme A
Contd...	:	Continued
CRD	:	Completely Randomized Design
dt.	:	Dated
DW	:	Distilled water
ELISA	:	Enzyme-Linked Immunosorbent Assay
<i>et al</i>	:	And other people

<i>etc</i>	:	And other
EU	:	European Union
FAO	:	Food and agriculture organization
FDA	:	Food and Drugs Administration
Fig	:	Figure
g	:	Gram/s
GRAS	:	Generally Recognized as Safe
Ha	:	Hectare
hrs	:	Hours
<i>i.e.,</i>	:	That is
IARC	:	International Agency for Research on Cancer
ICMR	:	Indian Council of Medical Research
ISO	:	International Organization for Standardization
kg	:	Kilogram
kg/ha	:	Kilograms per hectare
l	:	Liter
mg	:	Milligram
mha	:	Million hectare
min	:	Minutes
ml	:	Milliliter

mM	:	Milli molar
mm	:	Millimeter
mt	:	Million Tonnes
nm	:	Nanometer
No.	:	Number
OD	:	Optical Density
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
POD	:	Peroxidase
ppb	:	Parts Per Billion
ppm	:	Parts Per Million
psi	:	per square inch
RBC	:	Red Blood Cells
rpm	:	Rotations per minute
S.Em	:	Standard Error mean
Tons	:	Tonnes
U	:	Unit
<i>Viz.,</i>	:	Namely
WBC	:	White Blood Cells
WTO	:	World Trade Organization

I. INTRODUCTION

The International Organization for Standardization (ISO) defines an essential oil as a product made by distillation with either water or steam or by mechanical processing or by dry distillation of natural materials and appear as liquid, volatile, limpid and colored mixtures of several aromatic compounds. These are obtained from all plant parts, mainly from herbs and spices (Ravindran *et al.*, 2016 and Fengfeng *et al.*, 2017). About 3000 essential oils are known, 300 of which are commercially important, mainly used in the flavours and fragrances market (Burt *et al.*, 2003). The term “Essential Oil” was coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim. Plant essential oils were usually complex mixtures of natural compounds, both polar and non-polar (Masango., 2005 and Macwan *et al.*, 2016).

In nature, Essential oils play an important role in the protection of plants from pests. The role of essential oils is the protection of the plant against pathogenic microorganisms and insects. (Bakkali *et al.*, 2008).

The use of essential oils is common in earliest civilizations as growth promoters, food safety, feed additives, veterinary products, biopesticides, insecticides, repellents and fungicides in East, Middle East, North Africa and Europe (Macwan *et al.*, 2016). These are also used as antiseptic and medicinal properties (analgesic, sedative, anti-inflammatory, spasmolytic, local anaesthetic, anti-carcinogenic), embalmment and due to their antimicrobial and antioxidant activity, as natural additives in foods and food products (Burt *et al.*, 2003; Bakkali *et al.*, 2008; Tongnuanchan *et al.*, 2014; Mijat *et al.*, 2017).

Essential oils as promising natural products for fungal inhibition (Kalemba *et al.*, 2003; Hu *et al.*, 2007; Bakkali *et al.*, 2008; Prakash *et al.*, 2012; Lang *et al.*, 2012; Ghalem., 2016). Essential oils, like the other phytochemicals, could attenuate the microbial growth and biofilm development through specific mechanisms (Hyldgaard *et al.*, 2012). Due to their extensive antimicrobial properties, many essential oils could be used for the control of microbial spoilage, the preservation of food quality and safety, and the prolongation of their shelf life (Fратиanni *et al.*, 2010). Essential oils are classified as “Generally Recognized as Safe” (GRAS) by the Food and Drugs Administration (FDA), thus they are not harmful and, due to their natural origin, are more widely accepted by consumers than “synthetic” agents (Edris *et al.*, 2007).

Aspergillus flavus is a pathogenic fungus in the phylum Ascomycota. This species is known primarily for its ability to produce a potent toxin and carcinogen known as aflatoxin. Aflatoxin is known to contaminate many types of crop seeds, but in the field it is predominantly problematic for maize, peanuts, cotton seed and tree nuts. *A. flavus* also has a great impact on human health, in which immunosuppressed people are most susceptible to infection by this fungus. *A. flavus* is most common in warm temperate zones and environments with low water levels and higher temperatures (Klich., 2007).

A. flavus is found globally as a saprophyte in soils and causes disease on many important agriculture crops. Common hosts of the pathogen are cereal grains, legumes, and tree nuts. Specifically, *A. flavus* infection causes ear rot in corn and yellow mold in peanuts either before or after harvest. Infection can be present in the field, preharvest, postharvest, during storage and during transit. *A. flavus* has the potential to infect seedlings by sporulation on injured seeds. In grains, the pathogen can invade seed embryos and cause infection, which decreases germination and can lead to infected seeds planted in the field.

Rhizoctonia is a genus of anamorphic fungi in the order Cantharellales. Species do not produce spores, but are composed of hyphae and sclerotia and are asexual states of fungi in the genus *Thanatephorus*. *Rhizoctonia* species are saprotrophic, but are also facultative plant pathogens, causing commercially important crop diseases. In the absence of the host crop, it survives in soil as a competitive saprophyte on available dead organic matter.

R. bataticola is a polyphagous soil borne pathogen infecting over 500 plant species worldwide causing huge losses. Though the fungus is seed and soil borne inoculum (Dingra and Sinclair 1994). *R. bataticola* causes a wide range of commercially significant plant diseases. It is one of the fungi responsible for Dry root rot of chick pea, Brown patch (a turf grass disease), damping off in seedlings, as well as black scurf of potatoes, root rot of sugar beet, belly rot of cucumber, sheath blight of rice, and many other pathogenic conditions (Roberts., 1999).

Chemical control of dry root rot is not effective as *R. bataticola* has a broad host range and survives in soil for longer periods in the form of sclerotia. The sclerotia can survive up to 10 months even in the absence of the host plants and under prevailing dry soil conditions.

Essential oils and their components have a variety of targets, particularly the membrane and cytoplasm, and in certain situations, they completely alter the morphology of the cells (Nazzaro *et al.*, 2013). Both humans and plants are susceptible to fungal infections by pathogenic fungi and some synthetic fungicides are known to be effective in their control. However, the use of synthetic fungicides is limited by the emergence of resistant fungus strains and some fungicides possess considerable toxicity. Moreover, there is a growing public concern over the increased health and environmental hazard associated with synthetic molecules. For this reason, alternative, safe and natural methods to develop new antifungal agents are actively studied (Lopez-Reyes *et al.*, 2013). Recently, there has been a great interest in using essential oils as possible natural substitutes for conventional synthetic fungicides (Elshafie *et al.*, 2015).

The present investigations were undertaken to screen essential oils, plant extracts and synthetic chemicals to understand the ability of inhibiting *Aspergillus flavus* and *Rhizoctonia bataticola*.

Objectives

1. Screening of synthetic chemicals against *Aspergillus flavus* and *Rhizoctonia bataticola*.
2. Evaluation of essential oils and plant extracts against *Aspergillus flavus* and *Rhizoctonia bataticola*.
3. Study on effect of essential oils, plant extracts on cell integrity of the pathogens.

II. REVIEW OF LITERATURE

Most of the crop plants are attacked by seed and soil borne diseases. Among those pathogenic fungi, *Aspergillus flavus* and *Rhizoctonia bataticola* are known to infect and causing heavy losses, the disease severity depends upon the temperature and moisture conditions. For the management of *A. flavus* and *R. bataticola* synthetic fungicides are known to be effective. However, the use of synthetic fungicides is limited by the emergence of resistant fungus strains and some fungicides possess considerable toxicity. Moreover, there is a growing public concern over the increased health and environmental hazards associated with synthetic molecules. For this reason, in this study the emphasis is given to the essential oils and plant extracts for the management of *A. flavus* and *R. bataticola*.

The review of literature pertaining to the study of screening of plant extracts and essential oils for the management of *A. flavus* and *R. bataticola* is reviewed under the following headings.

2.1. *Aspergillus flavus*

2.2. *Rhizoctonia bataticola*

2.3. Resazurin assay

2.4. Pyocyanin assay

2.5. Peroxidase and Catalase assay

2.1. *Aspergillus flavus*

2.1.1. Taxonomic position of *A. flavus*

Kingdom: Fungi, Phylum: Ascomycota, Subphylum: Pezizomycotina, Class: Eurotiomycetes, Subclass: Eurotiomycetidae, Order: Eurotiales, Family: Trichocomaceae, Genus: *Aspergillus*, Species: *flavus*.

2.1.2. Symptomology

A. flavus colonies are commonly powdery masses of yellow-green spores on the upper surface and reddish-gold on the lower surface. In both grains and legumes, infection is minimized to small areas, and discoloration and dullness of affected areas is often seen.

Growth is rapid and colonies appear downy or powdery in texture. The conidiophores of *A. flavus* are rough and colorless. Phialides are both uniseriate and biseriata (Verghese., 2004).

Hyphal growth usually occurs by thread-like branching and produces mycelia. Hyphae are septate and hyaline. Once established, the mycelium secretes degradative enzymes or proteins which can break down complex nutrients (food). Individual hyphae strands are not typically seen by the unaided eye; however, conidia producing thick mycelial mats are often seen. The conidiospores are asexual spores produced by *A. flavus* during reproduction (Alexopoulos., 1996; Horn *et al.*, 2009 and Michelek *et al.*, 2010).

Recently, *Petromyces* was identified as the sexual reproductive stage of *A. flavus*, where the ascospores develop within sclerotia (Amaiike and Nancy., 2011). The sexual state of this heterothallic fungus arises when strains of opposite mating type are cultured together (Horn *et al.*, 2009). Sexual reproduction occurs between sexually compatible strains belonging to different vegetative compatibility groups.

A. flavus is complex in its morphology and can be classified into two groups based on the size of sclerotia produced. Group I consists of L strains with sclerotia greater than 400 µm in diameter. Group II consists of S strains with sclerotia less than 400 µm in diameter. Both L and S strains can produce the two most common aflatoxins (B1 and B2). Unique to the S strains is the production of aflatoxin G1 and G2 which typically are not produced by *A. flavus* (Horn *et al.*, 2009). The L strain is more aggressive than the S strain, but produces more less aflatoxin. The L strain also has a more acidic homeostatic point and produces less sclerotia than the S strain under more limiting conditions (Cotty., 1989).

2.1.3. Disease cycle

A. flavus overwinters in the soil and appears as propagules on decaying matter, either as mycelia or sclerotia. Sclerotia germinate to produce additional hyphae and asexual spores called conidia. These conidia are said to be the primary inoculum for *A. flavus*. The propagules in the soil, which are now conidia, are dispersed by wind and insects (such as stink bugs or lygus bugs). The conidia can land on and infect either grains or legumes. The spores enter the corn through the silks and thus infect the kernel. Conidiophores and conidia are produced in the spring from sclerotial surfaces. There is a secondary inoculum for *A.*

flavus, which is conidia on leaf parts and leaves. *A. flavus* grows on leaves after damage by leaf-feeding insects. Insects are said to be a source of inoculum and promote inoculum production (Diener *et al.*, 1987 and Hedayati *et al.*, 2007).

2.1.4. Use of natural products to control *Aspergillus* infection

Chemical control remains the main measure to reduce the incidence of post-harvest diseases in various foods. Antimicrobial chemicals belonging to the groups of benzimidazoles, aromatic hydrocarbons, and inhibitors of sterol biosynthesis are often used as post-harvest treatments. However, the application of high concentrations increases the risk of toxic residues in the products (Baird *et al.*, 1991; AL-Omair and Helaleh, 2004; Simko, 2005). Therefore, there has been increased interest in research on using natural antifungal substances, which may replace synthetic fungicides or contribute to the development of new disease control agents.

During the past 22 years, some essential oils have been shown to possess a broad spectrum of antifungal activity (Thompson, 1989 and Tian *et al.*, 2011). Screening experiments with 41 aqueous and ethanolic extracts and 22 essential oils against *Aspergillus* section Flavi strains have shown boldo, poleo, clove, anise and thyme oils as potential antifungal candidates (Bluma *et al.*, 2008). In their study, Essential oils were screened for antifungal effect by direct addition to and diffusion in the media. However, recent studies have shown that smaller compounds such as monoterpenes are most efficient when used as headspace volatiles (Avila-Sosa *et al.*, 2011). This characteristic makes essential oils attractive as possible fumigants for the protection of stored products.

The antimicrobial efficacy of clove oil treatment of groundnuts at 50 and 100 $\mu\text{l/ml}$ caused significant reductions in the *A. flavus* population compared to the control. The maximum reductions of *A. flavus* were reported as 6.7 \log_{10} times on groundnuts treated with 50-100 $\mu\text{l/ml}$ clove oil. In addition, *A. flavus* recovery after 3 days of storage was not detected. Three constituents, eugenol (89.8 per cent), caryophyllen (4.7 per cent) and vanillin (2.9 per cent) representing 98.4 per cent of the clove oil were identified. Clove oil suspensions can be used to enhance the microbial safety of groundnuts (Narumol and Jantamas, 2014).

Syzygium aromaticum (clove) is widely cultivated in Indonesia, Sri Lanka, Madagascar, Tanzania and Brazil. Previous studies has shown antifungal activity of clove oil and eugenol against yeasts and filamentous fungi, on several food-borne fungal pathogens (Lopez *et al.*, 2005) and human pathogenic fungi (Gayoso *et al.*, 2005 and Chaieb *et al.*, 2007). *S. aromaticum* active ingredients of cinnamaldehyde and eugenol are noted as antifungal components against filamentous soil and seed borne fungi (Paranagama., 1991 and Jayaratne *et al.*, 2002). Clove oil and eugenol have also been tested as antifungal agents in animal models (Chami *et al.*, 2004 and Ahmad *et al.*, 2005). In order to further clarify the spectrum of antifungal activity and its relationship to chemical composition, some general considerations must be established regarding the study of the antimicrobial activity of essential oils and the compounds isolated from them. Of the highest relevance is the definition of common parameters, such as the techniques employed, growth medium and micro-organisms tested. Standardization of both the methods of analysis of the essential oils and the assays for *in vitro* testing is required so that research in this area can be systematic and objective and the interpretation of results validated. The limited knowledge concerning antimicrobial activity and the mechanism of action of plant extracts has led to the addressing of such issues, although the main antifungal action of phenolic compounds, such as eugenol, appears to be exerted on the cellular membrane (Cox *et al.*, 2001 and Carson *et al.*, 2006).

Antifungal investigations revealed that garlic extract was effective against oilseed-borne toxigenic *Aspergillus* and *Penicillium* species (Ikeura *et al.*, 2011 and Tagoe *et al.*, 2011). In general, garlic has been found to have potential antifungal properties (Pereira *et al.*, 2006; Kanan and Najar., 2008). Moreover, in a study by Muhsin *et al.*, (2001) growth of 18 different fungal species was effectively inhibited by crude garlic bulb extract. Antifungal activity of garlic juice could be attributed to its phytochemical properties (Obagwu., 2003). Garlic allicin decomposes into several effective compounds, such as diallylsulphide, diallyldisulphide, diallyltrisulphide, allyl methyl trisulphide, dithiins and E,Z-ajoene, that serve as antimicrobial agents (Jabar and Mossawi., 2007). Inhibitory effects of garlic juice against *Aspergillus* and *Penicillium* fungi suggest the possible use of garlic in controlling food-spoiling fungi. Meanwhile, the use of water-based juice provides an alternative to chemical solvents, which can be toxic at certain concentrations. Garlic juice was capable of inhibiting fungal growth, and it can be used as a source of antifungal compounds to prevent fungal infections of stored groundnuts (Tagoe *et al.*, 2011).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a major constituent of vanilla bean, an orchid (*V. planifolia*, *V. pompona* or *V. tahitensis*). Vanilla is widely used in flavoring materials worldwide and is the second most expensive spice in the world next to saffron (Lubinsky *et al.*, 2008). Despite its broad utilization it had not been seriously researched for any bioactivity. However, it has been reported by some few researchers that vanilla might possess antimicrobial activity (Beuchat and Golden., 1989). Jay and Rivers (1984) found that vanilla was very active in suppressing moulds and non-lactic Gram positive bacteria. Lopez-Malo *et al.*, (1995) investigated with different fruit based agar media containing mango, papaya, pineapple, apple and banana with 2000 µg/ml vanillin and incubated each with *A. flavus*, *A. niger*, *A. ochraceus*, or *A. parasiticus*. Vanillin concentration at 1500 µg/ml significantly inhibited all the strains of *Aspergillus* in all media. However, vanillin had less effectiveness in banana and mango agars. Vanillin has also been reported to possess anti-clastogenic, anti-mutagenic and anti-tumour properties and, therefore, it can be considered as a nutraceutical molecule (Sinigaglia *et al.*, 2004 and Shyamala *et al.*, 2007). The antimicrobial property of vanillin is the effect of a phenolic compound which makes vanillin effective in inhibiting bacteria, yeasts and moulds. It is structurally similar to eugenol (2-methoxy-4-(2-propenyl) phenol) from clove and is known to be antimycotic (Beuchat and Golden., 1989) and bacteriostatic (Fitzgerald *et al.*, 2004). At low concentrations, phenols affect enzyme activity, especially those enzymes associated with energy production, while at greater concentrations they cause proteins to denature (Prindle and Wright., 1977). In other work on *Aspergillus* infection of groundnut, Mondali *et al.*, (2009) reported the efficacy of aqueous and alcoholic extracts of neem leaf, garlic, ginger and onion against seed-borne *A. flavus*, which showed that treatments were effective in inhibiting the pathogen. Srichana *et al.*, (2009) screened the efficacy of betel leaf extract on the growth of *A. flavus*, and it was shown that the extract at 10,000 ppm concentration was highly significant in suppressing the tested pathogen. This, however, is an extremely high concentration and the commercial viability of this material must be questioned.

Chatterjee (1990) showed that cassia, clove star-anise, geranium, and basil prevented the infection of maize seed *in vitro* by *Aspergillus flavus*, *A. glaucus*, *A. niger*, and *A. sydowi*. The seeds were artificially inoculated with the pathogens and treated with the essential oils at rates from 0 to 50 µl/g of grain. The author defined “the effective level” of the essential oils to the rate capable of controlling the pathogens. Even though the five

essential oils controlled the artificially inoculated fungi, the essential oils were unable to control other fungi already present on the seed.

Bansal and Sobti (1990) reported that neem extract was most effective plant extract in reducing the *A. flavus* incidence on groundnut seeds by application of two per cent aqueous solution of neem. The incidence of *A. flavus* on groundnut seeds was 2.66 per cent as compared to control with 9 per cent.

Srivatsava *et al.* (1997) studied the antifungal activity of neem (*Azadirachta indica* L.) and karanj (*Pongamia pinnata* L.) seeds and neem leaves against *A. flavus* and *A. niger*. Among different treatments, neem seeds treated with neem seed oil (NSO) was found best in inhibiting the mycoflora of neem seeds. The bioefficacy of neem seed kernel powder (NSKP), karanj seed kernel powder (KSKP) and neem leaf powder (NLP) were more effective when pelleted on wet seeds as compared to the dry seeds. The NSO and NLP inhibited the growth of *A. flavus*.

Montes-Belmont and Carvajal (1998) evaluated the fungicide properties of *Cinnamomum zeylanicum*, *Mentha piperita*, *Ocimum basilicum*, *Origanum vulgare*, *Teloxysam brosioides*, *Syzygium aromaticum*, and *Thymus vulgaris*. These plant essential oils controlled *A. flavus* growth on artificially infected maize seeds germinated in the laboratory. Corn seedlings treated with these essential oils did not exhibit phytotoxicity.

The essential oils of 12 medicinal plants viz. anise (*Pimpinella anisum*), caraway (*Carum carvi*), fennel (*Foeniculum vulgare*), thyme (*Thymus vulgaris*), spearmint (*Mentha spicata*), basil (*Ocimum basilicum*), chamomile (*Chamomilla recutita*), marigold (*Calendula officinalis*), hazanbul (*Achillea millefolium*), qyssum (*A. fragrantissima*), ghafath (*Agrimonia eupatoria*) and cinnamon (*Cinnamomum zeylanicum*) were tested for inhibitory activity against *Aspergillus flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium moniliforme*. The oils of thyme and cinnamon (≤ 500 ppm), marigold (≤ 2000 ppm), spearmint, basil and qyssum (3000 ppm) completely inhibited all the tested fungi. Caraway was inhibitory at 2000 ppm against *A. flavus* and *A. parasiticus*, and at 3000 ppm against *A. ochraceus* and *F. moniliforme*. *A. flavus*, *A. ochraceus*, *A. parasiticus* and *F. moniliforme* were completely inhibited by anise ≤ 500 ppm. However, chamomile and hazanbul at all concentrations were partially effective against the test toxigenic fungi. The results indicate that the test toxigenic fungi are sensitive to the 12 essential oils, and particularly sensitive

to thyme and cinnamon. The results also showed that the essential oils of thyme, cinnamon, anise and spearmint have more effect on fungal development and subsequent mycotoxin production in wheat grains. The extent of inhibition of fungal growth and mycotoxin production was dependent on the concentration of essential oils used (Soliman and Badea., 2002).

Paranagama *et al.* (2003) developed a natural fungicide against aflatoxigenic fungi, using the essential oil of lemongrass. They isolated *Aspergillus flavus* from stored rice and identified as aflatoxigenic grain. Lemongrass was tested against *A. flavus* and the test oil was fungi static and fungicidal against the test pathogen at 0.6 and 1.0 mg/ml, respectively. The results obtained from the thin layer and gas chromatography indicated citral A & B as fungicidal constituents in lemon grass oil. During the fumigant toxicity assay of lemongrass oil, the sporulation and the mycelia growth of the test pathogen were inhibited at the concentrations of 2.80 and 3.46 mg/ml respectively. Thus they concluded that lemongrass oil could be used as anti-fungal agent to manage aflatoxin formation and growth of *Aspergillus flavus* in stored rice.

Five essential oils extracted from *Cymbopogon citratus*, *Monodora myristica*, *Ocimum gratissimum*, *Thymus vulgaris* and *Zingiber officinale* were investigated for their antifungal effect against food spoilage and mycotoxin producing fungi, *Fusarium moniliforme*, *Aspergillus flavus* and *A. fumigatus*. Five strains of each fungus were tested. The essential oils from *O. gratissimum*, *T. vulgaris* and *C. citratus* were the most effective at 800, 1000 and 1200 ppm, respectively. Moderate activity was observed for the essential oils from *Z. officinale* between 800 and 2500 ppm, while the essential oil from *M. myristica* was less inhibitory (Nguefack *et al.*, 2004).

The oils of rosewood, bay, sassafras, and cinnamon inhibited the growth of *Aspergillus sp.*, *Fusarium sp.*, and *Penicillium sp. in vitro* (Simic *et al.*, 2004). Cinnamon essential oil was the most effective at controlling the pathogen growth, while bay oil was the least. GC-MS characterization showed that the essential oils had different composition. The authors concluded that the antifungal activity of the oils was based on the interactions among the different compounds, rather than a few compounds providing the antifungal properties to all oils.

Reddy *et al.* (2004) evaluated different botanicals/ plant extracts for the inhibition of fungal growth of *A. flavus*. Among the different plant extracts *Allium sativum* (bulb), *Azadirachta indica* (leaf), *Eucalyptus teriticornis* (leaf), *Pongamia pinnata* (kernel) extracts and garlic bulb extract (5%) completely inhibited the fungal growth of *A. flavus*. Pongamia kernel extract gave control of 57 per cent of *Aspergillus flavus* at 20 per cent concentration. Eucalyptus (40%) and neem extract (28%) were less effective in inhibiting the growth of *A. flavus*.

Ajith Kumar *et al.* (2005) reported 100 per cent inhibition of *A. flavus* by use of neem seed kernel extract, nimbidin and pongamia oil at 5 per cent concentration under *in vitro* in chilli.

Extract from *Garcinia pedunculata*, with a high content of phenolic acids, inhibited the growth and aflatoxin production in *A. parasiticus* and *A. flavus*. The extract from *Garcinia*, have a rich source of phenolic acids that inhibited more aflatoxin production than growth of *Aspergillus* section Flavi (Joseph *et al.*, 2005).

Pawar and Thaker (2006) screened seventy-five essential oils for their antifungal properties against *Aspergillus niger*. They reported that only five of the seventy-five oils controlled pathogen growth. Essential oils extracted from *Cinnamomum zeylanicum* (bark and leaf), *Cinnamomum cassia*, *Syzygium aromaticum*, and *Cymbopogon citratus* had good antifungal activity, while cinnamon bark oil had the best control rate.

Satish *et al.* (2007) studied the aqueous extract of 52 plants from different families for their antifungal potential against eight important species of *Aspergillus*. Among 52 plants tested, aqueous extract of *Acacia nilotica*, *Achras zapota*, *Datura stramonium*, *Emblica officinalis*, *Eucalyptus globules*, *Lawsonia inermis*, *Mimusops elengi*, *Peltophorum pterocarpum*, *Polyalthia longifolia*, *Prosopisjuliflora*, *Punica granatum* and *Syzygium cumini* have very good antifungal activity against one or the other *Aspergillus* species tested. Among the solvent extracts tested, methanol gave more effective than ethanol, chloroform, benzene and petroleum ether.

Plant essential oils are potential food preservatives due to their inhibitory effects on bacterial and fungal growth. Antifungal activities of *Thymus vulgaris* common thyme essential oil were tested against endophytic fungi grown from wheat (*Triticum aestivum*) grain, molecularly identified as *Alternaria alternata*, *A. infectoria*, *A. flavus*, *Epicoccum*

nigrum and *F. poae*. Treatment combinations of prior grain surface sterilization with hypochlorite and direct/indirect treatment with the essential oil were used, which showed strong effects on infection incidence and germination. Direct soaking of the wheat grain in the essential oil was particularly effective, but inhibited both fungal growth and seed germination (Kumar *et al.*, 2008).

The essential oils from 12 medicinal plants were tested to inhibit the *Aspergillus parasiticus*. The fungus was cultured in presence of various oils in 6-well microplates using a micro bioassay technique. The mycelial growth was estimated. Among essential oils tested, *Thymus vulgari* and *Citrus aurantifolia* were found to inhibit both *A. parasiticus*. The essential oils from *Mentha spicata* L., *Foeniculum miller*, *Azadirachta indica* A. Juss, *Conium maculatum* and *Artemisia dracunculus* were also inhibited fungal growth but not as *Thymus vulgari* and *Citrus aurantifolia*. The other plants including *Ferula gummosa*, *Citrus sinensis*, *Mentha longifolia* and *Eucalyptus camaldulensis* had no effect on *A. parasiticus* growth at all concentrations used. These results indicate that the essential oils of some medicinal plants may be considered as potential candidates to protect foods and feeds from toxigenic fungus growth (Razzaghi-Abyaneh *et al.*, 2009).

Mycotoxigenous fungi *Fusarium graminearum* and *Aspergillus flavus* were producing mycotoxins in the cereal grains. The antifungal action of the thyme essential oil on these two fungi was tested through diffusion in the culture medium Sabouraud with chloramfenicol in Petri plates in three doses: 1 µl, 5 µl and 10 µl. The obtained results in this study were evidencing the capacity of thyme oil on the inhibition of the development of the *F. graminearum* and *A. flavus*. On the media treated with essential oil in different doses the number of the colonies was very low (between 1 and 7) in comparison with the control tester were growth between 31 and 38 mycelial colonies. Inhibitory capacity of the thyme on *F. graminearum* and *A. flavus* was proved to be very good. The present researches show that in the antifungal activity are implied the fenolic compounds carvacrole and thymole. The very good antifungal capacity of the thyme volatile oil recommends it for the use in the control of some storage pathogens that cannot be kept under control through other methods (Fernanda *et al.*, 2012).

Adjou *et al.* (2012) studied antifungal activity of *Ocimum canum* essential oil against toxicogenic fungi. They evaluated the inhibition of *Aspergillus flavus* and *A. parasiticus* isolated from peanut and their aflatoxin production exposed to the essential oils

extracted from fresh leaves of *Ocimum canum*. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the oil were determined. The essential oil was found to be strongly fungicidal and inhibitory to aflatoxin production. Through GC/MS analysis, an amount of 30 components were identified, representing almost 95.2% of the oil. Essential oil of *O. canum* was characterized by major components such as terpinene-4-ol (41.18 %), linalol (14.7 %) and terpene (6.9 %). This plant offers novel approach to the management of storage fungi.

Passone *et al.* (2012) reported on the antifungal activity of five plant essential oils: from boldo *Peumus boldos* Molina, Poleo *Lippia turbinata* var. *integrifolia*, (Griseb.), clove *S. aromaticum* L., anise *Pimpinella anisum* and thyme *Thymus vulgaris*. These were tested against aflatoxigenic strains of *A. flavus* and *A. parasiticus* on groundnut-based media, conditioned at different water activities of 0.98, 0.95, 0.93. The effects of EOs were assessed, when the oils were applied to groundnut meal extract agar, by recording the lag phase, growth rate, and aflatoxin B1 accumulation of the tested pathogens. The results showed that lowest concentration (500 ppm) had no effect on the pathogens, but higher concentrations (2500 μl^{-1} for boldo and poleo; 1500 μl^{-1} for clove) completely inhibited the growth of *Aspergillus* spp. irrespective of the medium.

Sudha *et al.* (2013) evaluated the effect of different plant extracts *viz.*, neem (*Azadirachta indica*) seed kernel extract (NSKE), Pongamia (*Pongamia pinnata*) oil and nimbidin and rakshak at 1.0, 2.5 and 5.0 per cent concentration against *A. flavus* in chilli using the poisoned food technique. Cent % inhibition (100 %) of *A. flavus* was reported with NSKE, nimbidin and pongamia oil and least inhibition of growth in rakshak (93.3 %) at 5 per cent.

Srilakshmi *et al.* (2013) investigated the bioactivity of secondary metabolites or small molecules produced by *Trichoderma* spp. and their efficacy against aflatoxin contamination in groundnut. The results indicated that 48 strains of *Trichoderma* were highly significant in suppressing an *A. flavus* isolate (AF11-4) and subsequently reduced aflatoxin production in groundnut. It is also feasible to apply BCAs in combination, sometimes including bacterial and fungal antagonists.

The antimicrobial activity of essential oils depends on the combination and proportions of different compounds found in their composition. Monoterpenes are the main constituents of essential oils, and many of them have been reported for their antifungal, anti-aflatoxin and antioxidant activity. Studies showed that essential oils possess antifungal activity being able to inhibit the aflatoxin production (Kedia *et al.*, 2014). The antifungal activity of essential oils was studied and reported using different methods as there are no standard protocols to test the effect of these naturally occurring compounds on fungi.

2.2. *Rhizoctonia bataticola*

2.2.1. Taxonomic position of *R. bataticola*

Kingdom: Fungi, Phylum: Basidiomycota, Subphylum: Aquaricomycotina, Class: Basidiomycetes, Subclass: Aquaricomycetidae, Order: Polyporales, Family: Corticiaceae, Genus: *Rhizoctonia*, Species: *bataticola*.

2.2.2. Symptomology

Philip *et al.* (1969) and Grover and Sakhuja (1981) reported leaf blight phase of mung bean with the information that disease usually makes its appearance when the crop is 4-6 weeks old and the older leaves are first affected. Initially small, circular to irregular brown to reddish brown lesions appear on or near the margins which enlarge and coalesce. Under hot and humid conditions the entire plant may be blighted. The dried lesions are yellowish brown and become papery in texture. Severely affected leaves fall off prematurely.

Nene *et al.* (1978) observed continuous black discoloration of pith and xylem vessels of roots and basal shoots as one of the main characteristic symptoms of dry root rot of chickpea. Sanecha and Srivastava (1982) reported that in affected young seedlings of cowpea, discoloration and rotting occurred from young root tip and proceed backwards. The cotyledonary leaves are completely blighted and necrotic. Stem decay occurs in advanced stage

Baldev *et al.* (1988) reported symptoms of dry root rot of chickpea which consisted of straw colored leaves and stems. Tap root of infected plants was observed to be dry and devoid of lateral and finer roots. Roots were turned dark and showed signs of rotting. The

dead root was observed brittle towards the tip and showed shredding of bark. Haware (1990) reported that dry root rot of chickpea appears around flowering and podding time in the form of scattered dried plants but seedlings are also infected. The symptoms induced were drooping of petioles and leaflets which were confined to top of the plants. Shredding of bark in form of flakes was observed. Singh *et al.* (1990) observed that *R. bataticola* inoculated roots of chickpea, upon microscopic examination, showed disintegration of cortical tissue and plugging of xylem vessels with mycelial and sclerotial bodies of fungus.

Rangaswamy (1996) reported field symptoms which include yellowing of plants with drooping of leaves. Due to decay of roots, plants can be easily uprooted. Root portion appears brownish from outside. The stem and root below the region shows rotting with frequently pinkish white mycelial growth. Dried plants scattered throughout the field are indicative of root rot incidence.

Singh (1999) reported that dry root rot of chickpea occurred from flowering to podding stage. Infected plants were suddenly dried in the field. The tap roots turned dark brittle and devoid of lateral roots. Khalid and Ilyas (2000) considered the presence of root lesions and sclerotium plugging in xylem vessels of roots and collar region as symptoms of dry root rot of chickpea for screening of germplasm against this disease. Singh and Agarwal (2002) observed the withering and drying of chickpea plants in the field due to the infestation of *R. bataticola*.

2.2.3. Disease cycle

R. bataticola can survive for many years by producing small (1 to 3-mm diameter), irregular-shaped, brown to black structures (called sclerotia) in soil and on plant tissue. *R. bataticola* have evolved the ability to produce sclerotia with a thick outer layer that allows them to float and survive in water. *R. bataticola* also survives as mycelium by colonizing soil organic matter as a saprophyte, particularly as a result of plant pathogenic activity. Sclerotia and/or mycelium present in soil and/or on plant tissue germinate to produce vegetative threads (hyphae) of the fungus that can attack a wide range of food and fiber crops. The fungus is attracted to the plant by chemical stimulants released by actively growing plant cells and/or decomposing plant residues. As the attraction process proceeds, the fungal hypha will come in contact with the plant and become attached to its external surface. After attachment, the fungus continues to grow on the external surface of the plant

and will causes disease by producing a specialized infection structure (either an appressorium or infection cushion) that penetrates the plant cell and releases nutrients for continued fungal growth and development. The infection process is promoted by the production of many different extracellular enzymes that degrade various components of plant cell walls (e.g. cellulose, cutin and pectin). As the fungus kills the plant cells, the hyphae continue to grow and colonize dead tissue, often forming sclerotia. New inoculum is produced on or in host tissue and a new cycle is repeated when new substrates become available.

2.2.4. Use of natural products to control *Rhizoctonia* infection

Singh *et al.* (1980) studied the effect of aqueous extract and oil of neem (*Azadirachta indica*) on soil borne pathogen *R. bataticola*, causing dry root rot in chickpea (*Cicer arietinum*). Growth of pathogen, in liquid media, was inhibited by extracts of leaf, trunk, bark fruit pulp and oil. Out of them, neem oil showed maximum inhibitory effect.

Singh *et al.* (1980) and Bhaskar *et al.*, (2005) reported that neem (*Azadirachta indies*) leaf extracts effective in inhibiting the mycelial growth of *R. bataticola*.

Kishore *et al.* (1982) screened 31 plant species to evaluate their fungi toxicity against *R. bataticola*. The leaves of *Allamanda cathartics* and *Artabotrys hexapetalus* showed absolute toxicity against *R. bataticola*. Sindhan and Jaglan., (1988). Reported that effectiveness of neem as well as *Allium cepa* and *A. sativam* against *R. bataticola*.

Mishra and Tiwari (1992) tested the leaf extract of *Polyanthia longifolia* against five rice pathogens viz., *Pyricularia oryzae*, *Rhizoctonia solani*, *R. bataticola*, *Fusarium moniliforme*, *A. niger* and *Curvalaria sp.* and reported significant reduction in the mycelial growth of *R. bataticola* in *Polyanthia longitfolia*.

Ansari (1995) reported antifungal activity of ajowain (*Trachispermum ammi*), lemon grass (*Cymbopogon citratus*), Tulsi (*Ocimum sp.*), mentha (*Mentha sp.*), *Rauwolfia sp.*, mehandi (*Lawsonia inermis*), and samhalu (*Vertex trifolia*). All the medicinal plants significantly reduced the growth of *R. bataticola*.

Shivpuri *et al.* (1997) worked out the toxicity of extracts of ten plant species (*Allium sativam*, *Allium cepa*, *Azadirachta indica*, *Calotropis procera*, *Datura stramonium*,

Ocimum sanctum, *Polyanthia longifolia*, *Tagetes erecta*, *Vinca rosea* and *Withania somnifera* under laboratory conditions and found fungitoxic properties of these botanicals against *R. bataticola*.

Sindhan *et al.* (1999) tested nine plant extracts against mycelial growth of *Macrophomina phaseolina* and *Rhizoctonia solani* *in vitro* at 5, 10 and 20 per cent concentration. All plant extracts were found inhibitory to *Rhizoctonia solani* and *M. phaseolina* at all concentrations. Both fungi were found more sensitive to extracts of onion, ginger, neem, garlic followed by mint, eucalyptus and tulsi.

Kaushal *et al.* (2003) tested 24 botanicals belonging to the family *Compositae* for their fungitoxicity against *R. bataticola*. The highest percent inhibition of mycelial growth was observed at 1000 µg/ml followed by 200 µg/ml at 48 and 60 hrs respectively.

In vitro studies have demonstrated antibacterial activity of essential oils against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus* at levels between 0.2 and 10 µl/ml. Gram-negative organisms were slightly less susceptible than gram-positive bacteria. A number of essential oils components has been identified as effective antibacterials, e.g. carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid (Burt, 2004).

Sharma *et al.* (2005) have demonstrated the efficacy of plant extract of *Eucalyptus globulus* against *R. bataticola* on gram. There was 85 per cent reduction in sclerotial formation when ten per cent plant extract was used. Leaf extract of neem (*Azadirachta indica*) was found effective in inhibiting the mycelial growth of *R. bataticola* in chickpea (Kshirsagar *et al.*, 2004; Sharma *et al.*, 2005; Shahraj *et al.*, 2007).

Tandel *et al.* (2010) tried phyto-extracts of eleven plant species against *R. bataticola* of gram and revealed that the onion bulb extract produced maximum inhibition (98.14 %) followed by extract of acacia, ginger, neem, garlic and karanj.

Shahnaz *et al.* (2010) reported that *Macrophomina phaseolina* and *Rhizoctonia solani* of okra were completely suppressed when *Datura alba* extract used as soil drenching and seeds coated with *Paecilomyces variotii*.

Kumar *et al.* (2011) tested neem leaf extract (20 %) and found effective in inhibiting (55.6 %) mycelium growth of *R. bataticola*, causing root rot of jatropha.

Evaluating the effects of some essential oils *i.e.*, eucalyptus, lemon grass and thyme on linear growth and spore germination of pathogenic fungi. Moreover, their efficacy on gray and blue moulds incidence of apple fruits was tested. All treatments significantly reduced the linear growth and spore germination of both tested fungi. Essential oils of eucalyptus, lemon grass and thyme at a concentration of 0.6 and 0.8 per cent completely inhibit the linear growth and spore germination of *B. cinerea* and *Penicillium sp.* except that lemon grass at 0.6 per cent. Sterilized water containing essential oils of eucalyptus, lemon grass and thyme at different concentrations, *i.e.*, 0.0, 0.6 and 0.8 per cent (v/v) were tested to study their effect against gray and blue mould incidence of apple fruits. The most effective treatment was eucalyptus and thyme at 0.8 per cent, which reduced the disease incidence more than 83.8 and 82.7 per cent and rotted part tissue by 85.9 and 88.5 per cent for gray and blue moulds, respectively (Latif *et al.*, 2016).

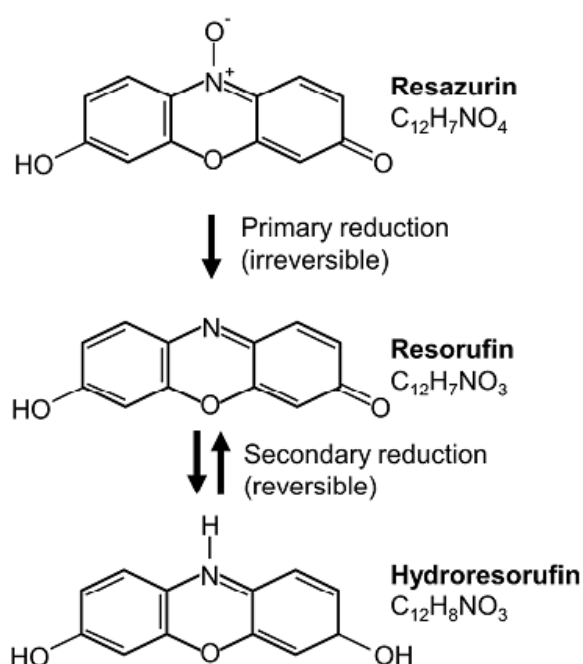
Evaluated the antifungal activity of essential oil of Eucalyptus (*Camaldulensis dehn*) against five *Fusarium spp.* commonly associated with maize. The essential oil produced complete mycelial growth inhibition in all the test pathogens at a concentration of 7-8 $\mu\text{l/ml}$ after five days of incubation. The minimum inhibitory concentration and minimum fungicidal concentration of the essential oil on the test fungi were in the range of 7-8 $\mu\text{l/ml}$ and 8-10 $\mu\text{l/ml}$, respectively. These findings confirm the fungicidal properties of *E. camaldulensis* essential oils and their potential use in the management of economically important *Fusarium spp.* and as possible alternatives to synthetic fungicides (Martin *et al.*, 2017).

2.3. Resazurin assay

Resazurin is a blue dye that is internalized by cells and metabolically reduced to the highly fluorescent pink compound resorufin (Fig 1) that is freely released from cells (Wilson *et al.*, 2000). The irreversible reduction of resazurin to resorufin is mediated by intracellular diaphorase enzymes (Zalata *et al.*, 1998 and Wilson *et al.*, 2000) and generates a strong fluorescent signal that may be measured using a spectrophotometer to noninvasively provide a comprehensive assessment of cellular metabolic activity within a population of cells. The resazurin reduction assay is inexpensive and non-toxic to cells at

low concentrations and brief incubation periods [i.e., <4 hours (Gloeckner *et al.*, 2001)], and is therefore a useful method to indirectly measure cell proliferation kinetics. Resazurin has been used to gauge cell number within bioengineered muscle scaffolds (Wolf *et al.*, 2012), lung matrices (Ren *et al.*, 2015 and Tapias *et al.*, 2015), and kidney scaffolds (Caralt *et al.*, 2015). The calculated viable cell number within a scaffold is determined from a standard curve in which a linear relationship is derived between cell number (on a per-volume basis) and fluorescence intensity (FI), following treatment of the examined cell population with a known volume of resazurin over a specific time-period (Wilson *et al.*, 2000, Ren *et al.*, 2015 and Gaalen *et al.*, 2010). However, the reliability of the calculated cell number using this method is dependent upon (1) a constant average metabolic activity across the cell population that does not change under the desired experimental conditions (i.e., at different evaluation time points) and (2) a constant resazurin reduction rate or, more specifically, a stable rate of FI increase.

Fig 1. Stepwise conversion of resazurin to resorufin and hydroresorufin. Resazurin (blue) diffuses into cells where it is irreversibly reduced by diaphorase enzymes to a highly fluorescent (pink) compound, resorufin. In a subsequent, reversible reaction, resorufin may be further reduced to colorless, non-fluorescent hydroresorufin (Zalata *et al.*, 1998 and Wilson *et al.*, 2000).



2.4. Pyocyanin assay

Quorum sensing controls the virulence determinants in most proteobacteria. In their study, the hexane, chloroform and methanol extracts of an Ayurveda spice, namely clove (*Syzygium aromaticum*), shown anti-quorum sensing activity. Hexane and methanol extracts of clove inhibited the response of *C. violaceum* CV026 to exogenously supplied *N*-hexanoyl homoserine lactone, in turn preventing violacein production. Chloroform and methanol extracts of clove significantly reduced bioluminescence production by *E. coli* [pSB1075] grown in the presence of *N*-(3-oxododecanoyl)-L-homoserine lactone. Thiba *et al.*, 2012 demonstrated that clove extract inhibited quorum sensing-regulated phenotypes in *Pseudomonas aeruginosa* PA01, including expression of *lecA::lux* (by hexane extract), swarming (maximum inhibition by methanol extract), Pyocyanin (maximum inhibition by hexane extract). This study shows that the presence of natural compounds that exhibit anti-quorum sensing activity in the clove extracts may be useful as the lead of anti-infective drugs.

Michelle *et al.* (2016) demonstrated protocol describes the quantification of pyocyanin extracted from swarming colonies of *Pseudomonas aeruginosa*. Pyocyanin is a secondary metabolite and a major virulence factor, whose production is inducible and varies highly under different growth conditions. The protocol is based on the earlier developed chloroform/HCl extraction of pyocyanin from liquid cultures (Frank and Demoss, 1959). Swarming colonies together with the agar they occupy are split into two halves. Pyocyanin is extracted from one of them. Cells are collected from the other half and used to quantify total protein yield and normalize the estimated corresponding pyocyanin quantities.

2.5. Peroxidase and Catalase assay

Leo *et al.* (1984) examined 40 strains of *Legionella* for reduced-oxygen scavenging enzymes. Using a simple reaction chamber with a Swinney filter for the Beers and Sizer assay, they determined the catalase activity of live cells grown on buffered charcoal-yeast extract agar. For 29 strains of *Legionella pneumophila*, the apparent first order rate constants for catalase ranged from 0.000 to 0.005. Similarly, low values ranging from 0.001 to 0.005 were observed for *Legionella wadsworthii*, *Legionella oakridgensis*, and *Legionella gormanii*. High catalase activities were found for *Legionella jordanis*,

Legionella longbeachae, *Legionella micdadei*, and *Legionella bozemanii*, with first-order rate constant values of 0.010 to 0.035. Cell-free extracts were analyzed for catalase, peroxidase, and superoxide dismutase. Cell-free extracts of all strains had superoxide dismutase levels ranging from 8.2 to 30.5 U per mg of protein.

III. MATERIAL AND METHODS

The research work was carried out during 2017-18 at the Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture and Main Agricultural Research Station, University of Agricultural Sciences Raichur, Karnataka (India). The campus is being geographically situated in the North Eastern Dry Zone (Zone 2) of Karnataka at 16°12" N latitude and 77° 21" E longitude with an altitude of 389.37 meters above mean sea level. The details of the material used and the methodology followed during the investigations are below.

3.1. General laboratory procedure

3.1.1. Glassware and cleaning

For all the laboratory experimental studies, Borosil and Technico glass wares were used. They were washed with detergent powder followed by washing in running tap water and then rinsed in distilled water and kept in hot air oven for few minutes for complete drying of moisture.

3.1.2. Sterilization

All glass wares, micropipette tips, solid and liquid culture media were subjected to sterilization by autoclaving at 121 °C, 15 psi for 20 min. The tip of inoculation loop was sterilized by using flame, all cultural studies were conducted under aseptic conditions in laminar flow chamber.

3.2. Source of pathogenic fungi

The identified pure culture of pathogenic fungi *A. flavus* and *R. bataticola* were collected from Department of Plant Pathology, College of Agriculture, UAS, Raichur for the study.

3.3. Maintenance of cultures

The pathogenic fungi *A. flavus* and *R. bataticola* cultures were subcultured on PDA slants and kept at 28 ± 2 °C for 7 days. Those slants were preserved in refrigerator at 4 °C and maintained by sub-culturing once in a month in order to avoid a decline in strain viability. Such cultures were used throughout the study.

3.4. Media composition and preparation

3.4.1. Potato dextrose agar

Peeled potato - 200 g

Dextrose - 20 g

Agar-agar - 20 g

Distilled water - 1000 ml

Preparation: Two hundred grams of peeled potatoes were cut into small pieces and boiled in distilled water and then extract was collected by filtering through muslin cloth. Dextrose 20 g and Agar 20 g each were dissolved in the potato extract and the final volume was made up to 1000 ml with distilled water and sterilized as described earlier and preserved for further use.

3.4.2. Potato dextrose broth

Peeled potato - 200 g

Dextrose - 20 g

Distilled water - 1000 ml

Preparation: Two hundred grams of peeled potatoes were cut into small pieces and boiled in distilled water and then extract was collected by filtering through muslin cloth. Dextrose 20 g was dissolved in the potato extract and the final volume was made up to 1000 ml with distilled water and sterilized as described earlier and preserved for further use.

3.5. Screening of Essential Oils

3.5.1. Collection of Essential Oils

Different essential oils were obtained from different companies as mentioned below.

Table 1. List of different essential oils and their companies

Sl. No.	Essential oil	Company	Place
1	Orange oil	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
2	Peppermint oil	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
3	Citronella oil	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
4	Methyl eugenol	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
5	Eugenol	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
6	Ajowain seed oil	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
7	Cumin seed oil	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
8	Rose oil	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
9	Lavender oil	Sisco Research Laboratories Pvt. Ltd.	Maharashtra
10	Clove oil	Avra Synthesis Pvt. Ltd.	Nacharam, Hyderabad
11	Cinnamon oil	Ajanta Aromas	Kannauj, Uttar Pradesh
12	Eucalyptus oil	James Perfumery co.	Mysore

3.5.2. Dilution of Essential Oils

Each essential oil was diluted at different concentrations such as 0.05, 0.1, 0.25, 0.5, 1 and 2 per cent in 10 ml of PDB and 10 μ l of Tween-20 (Sigma-Aldrich) to dissolve essential oil in water.

3.5.3. Screening of essential oils against *A. flavus* and *R. bataticola* by using Microtiter plate

A simple technique which enables the monitoring of fungal growth with the aid of a microplate reader was followed according to Willem *et al.* (1989). Sterile 96-well plate was labelled (Fig.2), Culture of fungi for the microplate reader assay was done in sterile flat bottom microtiter plates at 28 ± 2 °C. 300 μ l of distilled water was taken in 1st column, Controls (290 μ l of PDB + 10 μ l of *A. flavus*/*R. bataticola* culture) were maintained without essential oils in a 2nd column and Test samples with different concentrations of essential oils (290 μ l of PDB with essential oils + 10 μ l of *A. flavus*/*R. bataticola* culture) were maintained in next columns and performed in triplicates. All the microtiter plates were sealed with para film and incubated at 28 ± 2 °C. Microplate readings were taken in Micro plate reader (Bio-Rad) immediately after inoculation and for every 24 hr for 7 days at 595 nm.

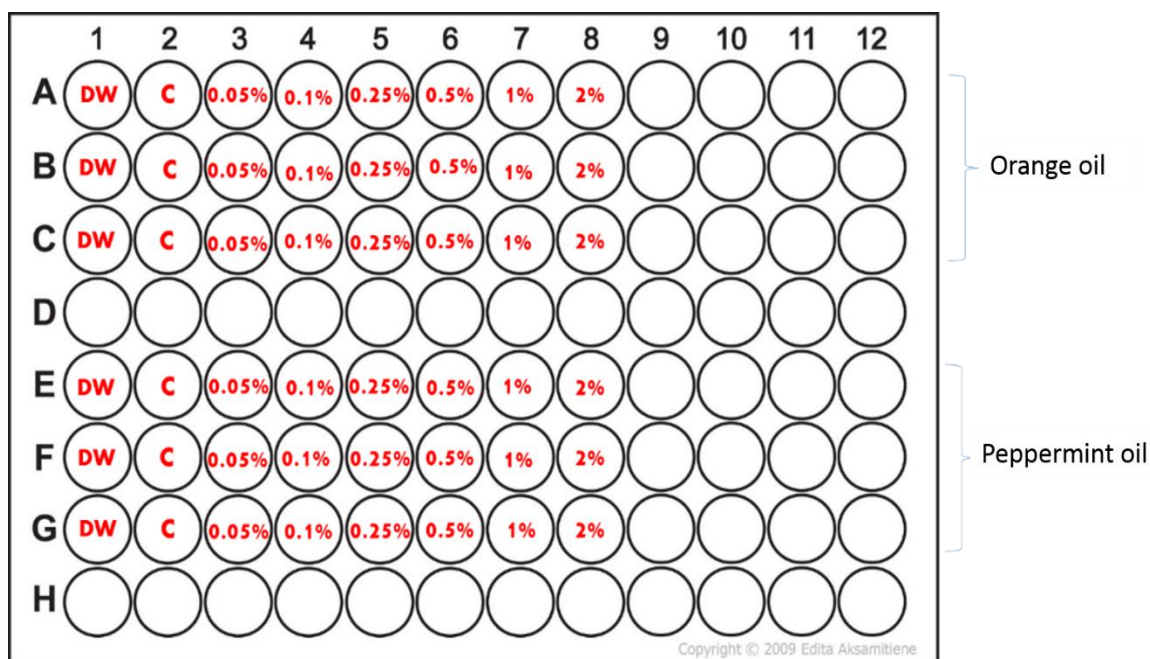


Fig 2. Figure showing 96-well plate format for microtiter plate

DW-Distilled water, C-Control

3.5.4. Screening of essential oils against *A. flavus* and *R. bataticola* by poison food technique

In vitro evaluation of essential oils against *A. flavus* and *R. bataticola*

The experiment was carried out in completely randomized design. The efficacy of essential oils each as mentioned in Table.1 were evaluated against *A. flavus* and *R. bataticola* under *in vitro* conditions on the PDA media using poison food technique (Nene and Thapliyal, 1993). The required quantities of individual essential oils were added separately into molten and cooled PDA to get the desired concentration (Control, 0.05 %, 0.1 %, 0.25 %, 0.5 %, 1 % and 2 %) of the essential oils. Later 2 ml of such poisoned medium was poured into sterile Petri plates. 10 µl of seven day old liquid culture of the *A. flavus/R. bataticola* was spotted on to the media at the center of the Petri plates. Control was maintained without adding any essential oil to the medium and each treatment was replicated thrice. Then such plates were incubated at 28 ± 2 °C temperature for seven days and radial colony growth (cm) was measured. The efficacy of an essential oil was expressed as per cent inhibition of mycelial growth over control that was calculated by using Vincent's formula (1947). The per cent values were converted into angular transformations and the data were analyzed statistically by FCRD analysis.

The fungi toxicity of the essential oils in terms of per cent inhibition of mycelial growth was calculated using the formula:-

$$I = \frac{C - T}{C} \times 100$$

Where

I = Per cent inhibition of mycelium

C = Growth of mycelium in control

T = Growth of mycelium in treatment

3.5.5. Resazurin assay for cell viability

3.5.5.1. Preparation of Resazurin solution

0.02 per cent of Resazurin solution was prepared by dissolving 2 mg in 10 ml of sterile distilled water. The solution was vortexed and dissolved to make homogenous solution.

3.5.5.2. Preparation of the plates

Plates were prepared under aseptic conditions. The plates were prepared in triplicate. A sterile 96-well plate was labelled (Fig 3). 180 μ l of diluted essential oil samples were loaded according to the label and 20 μ l of *A. flavus*/*R. bataticola* culture was added into all the wells, controls were maintained without essential oils. Sealed with para film and incubated at 28 ± 2 °C for 5 days. After 5 days of incubation 20 μ l of 0.02 per cent Resazurin solution was added into all wells and incubated for 30 min. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value (Bonnier *et al.*, 2015).

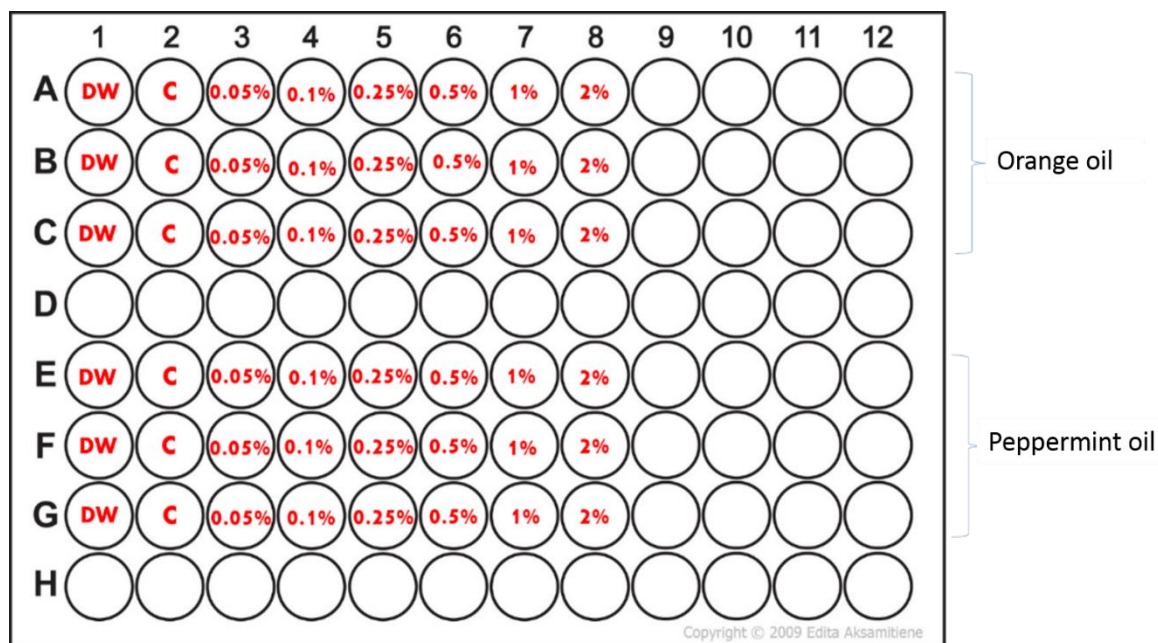


Fig 3. Figure showing 96-well format for Resazurin assay for essential oils

DW-Distilled water, C-Control

3.5.6. Pyocyanin assay

Pyocyanin was extracted from 5 days grown *A. flavus/R. bataticola* culture supernatant as previously described (Essar *et al.*, 1990). PDB media was prepared and *A. flavus/R. bataticola* culture was inoculated. Grown the *A. flavus/R. bataticola* cultures in treated (control, 0.5 %, 1 % and 2 % with 5 effective essential oils). Incubated at 28 ± 2 °C in shaker for 5 days. After the 5 days of growth, the supernatant was collected and 5 ml of supernatant from each flask was mixed with 3 ml of chloroform and mixed vigorously. The chloroform layer was mixed with 1ml of 0.2 M HCl. After centrifugation (10 min, 28 °C, 8,000 rpm), the OD of the HCl layer was measured at 520 nm against 0.2 M HCl using an UV/Vis spectrophotometer (Eppendorf).

3.5.7. Estimation of peroxidase (POX) activity

POX catalyzes the dehydrogenation of a large number of organic compounds as phenols and aromatic amines. It was determined following the dehydrogenation of guaiacol as a substrate according to Malik and Singh (1980).

Reagents

Phosphate buffer 0.1 M (pH 7.0)

Guaiacol solution 20 mM: Dissolve 240 mg guaiacol in water and make up to 100 ml. It can be stored frozen for many months.

Hydrogen peroxide solution (0.042 % = 12.3 mM): Dilute 0.14 ml of 30 % H₂O₂ to 100 ml with water, prepare freshly.

Preparation of enzyme extract: 6 days grown *A. flavus/R. bataticola* cultures were added with 1 % essential oils (Eugenol, Ajowain seed oil, Rose oil, Clove oil and Cinnamon oil) in different flasks. Collected 1.5 ml of sample at 4 hr, 12 hr and 24 hr after adding the essential oils. Centrifuged the culture at 1,000 rpm and collected the pellet. Homogenized the pellet in 1 ml of 0.1 M potassium phosphate buffer, pH 6.5 at 4 °C and centrifuged at 10,000 rpm for 10 min and the supernatant was collected and used for estimation of peroxidase activity.

Assay: Pipetted 1ml of the buffer solution, 0.2 ml guaiacol solution, 0.1 ml enzyme extract, 0.2 ml hydrogen peroxide solution and 0.5 ml distilled water to make up total volume of 2 ml in a cuvette. The mixture was shaken and placed in the spectrophotometer. The change in optical density at 0 min and 3 min at 470 nm was recorded and activity was calculated. Activity of peroxidase was calculated using the formula.

$$U/ml = \frac{\Delta OD \times RmV / \epsilon_{470} \times EV}{\text{Total protein content}}$$

Where, ΔOD = Change in absorbance,

RmV = Reaction mixture volume (ml)

ϵ_{470} = Molar absorptivity of H_2O_2 at 470 nm (6.375) and

EV = Enzyme extract volume (ml)

3.5.8. Estimation of catalase (CAT) activity

Catalase is a ubiquitous antioxidant enzyme that is present in nearly all living organisms. It catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Catalase is a tetramer of four polypeptide chains, and contains four porphyrin heme (iron) groups that allow the enzyme to react with hydrogen peroxide. Several pathogens produce catalase in order to defend themselves against attacks by hydrogen peroxide, a weapon commonly used by the host's immune system, in addition to oxidative stress.

Reagents

Phosphate buffer 0.1 M (pH 7.0)

Hydrogen peroxide solution 30 mM

Preparation of enzyme extract: 6 days grown *A. flavus*/*R. bataticola* cultures were added with 1 % essential oils (Eugenol, Ajowain seed oil, Rose oil, Clove oil and Cinnamon oil) in different flasks. Collected 1.5ml of sample at 4 hr, 12 hr and 24 hr after adding the essential oils. Centrifuged the culture at 1,000 rpm and collected the pellet. Homogenized the pellet in 1 ml of 0.1 M potassium phosphate buffer, pH 6.5 at 4 °C and centrifuged at 10,000 rpm for 10 min and the supernatant was collected and used for estimation of catalase activity.

Assay: Pipetted 1 ml of the buffer solution, 0.1 ml enzyme extract, 0.4 ml hydrogen peroxide solution and 0.5 ml distilled water in a cuvette. The mixture was well shaken and placed in the spectrophotometer. The change in optical density at 0 min and 3 min at 240 nm was recorded and used in calculations. Calculate the catalase activity using the following formula (Cuellar-Cruz *et al.* 2009).

$$U/ml = \frac{(A_{1min} - A_{3min}) \times V_t}{\epsilon_{240} \times d \times V_s \times C_t \times 0.001}$$

Where, $(A_{1min} - A_{3min})$ = Difference between the initial and final absorbance,

V_t = Total volume of the reaction (2 ml),

ϵ_{240} = Molar extinction coefficient for H_2O_2 at OD_{240} ($34.9 \text{ mol}^{-1} \text{ cm}^{-1}$),

d = Optical length path of cuvette (1 cm),

V_s = Volume of the sample in ml and

C_t = Protein concentration of the sample in mg/ml.

3.6. Screening of Plant Extracts

3.6.1. Collection of Plant Extracts

Methanolic plant extracts (24) were obtained from Jain University, Bangalore.

Table 2. List of plant extracts

Sl. No.	Plant Extracts (Code)	Code
1	<i>Azadirachta indica</i> leaf	1
2	<i>Centella asiatica</i>	2
3	<i>Ocimum gratissimum</i>	3
4	<i>Ocimum tenuiflorum</i>	4
5	<i>Aeonium arboreum</i>	5
6	<i>Styrax</i> spp.	6
7	<i>Laguncularia recemosa</i>	7
8	<i>Euphrasia officinalis</i>	8
9	<i>Tecoma stans</i>	9
10	<i>Duranta erecta</i>	10
11	<i>Urtica dioica</i>	11
12	<i>Morinda citrifolia</i>	12
13	<i>Justicia adhatoda</i>	13
14	<i>Vitex nigundo</i>	14
15	<i>Vinca major</i>	15
16	<i>Lawsonia inermis</i>	16
17	<i>Gloriosa superba</i>	17
18	<i>Lonicera japonica</i>	18
19	<i>Aloe vera</i>	20
20	<i>Baccharis trimera</i>	21
21	<i>Cordia verbenacea</i>	22
22	<i>Euphorbia tirucalli</i>	23
23	<i>Juglans regia</i>	24
24	<i>Leonotis nepetaefolia</i>	25

3.6.2. Dilution of Plant Extracts

Each plant extract was diluted at different concentrations such as 0.5, 1 and 2 per cent. Plant extract was directly added into 96-well microtiter plate.

3.6.3. Screening of plant extracts against *A. flavus* and *R. bataticola* by using Resazurin assay

3.6.3.1. Preparation of Resazurin solution

0.02 per cent of Resazurin solution was prepared by dissolving 2 mg in 10 ml of sterile distilled water. The solution was vortexed and dissolved to make homogenous solution.

3.6.3.2. Preparation of the plates

Plates were prepared under aseptic conditions. The plates were prepared in triplicate. A sterile 96-well plate was labelled (Fig.4). 180 μ l of PDB, 20 μ l of *A. flavus*/*R. bataticola* culture was added into all the wells and plant extracts were added into the labelled wells accordingly in 3 different concentrations (0.5, 1 and 2 %). i.e; 1 μ l, 2 μ l and 4 μ l for 200 μ l. Controls were maintained without plant extracts. Sealed with parafilm and incubated at 28 ± 2 °C for 5 days. After 5 days of incubation 20 μ l of 0.02 per cent Resazurin solution was added into all wells and incubated for 30 min. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value (Bonnier *et al.* 2015).

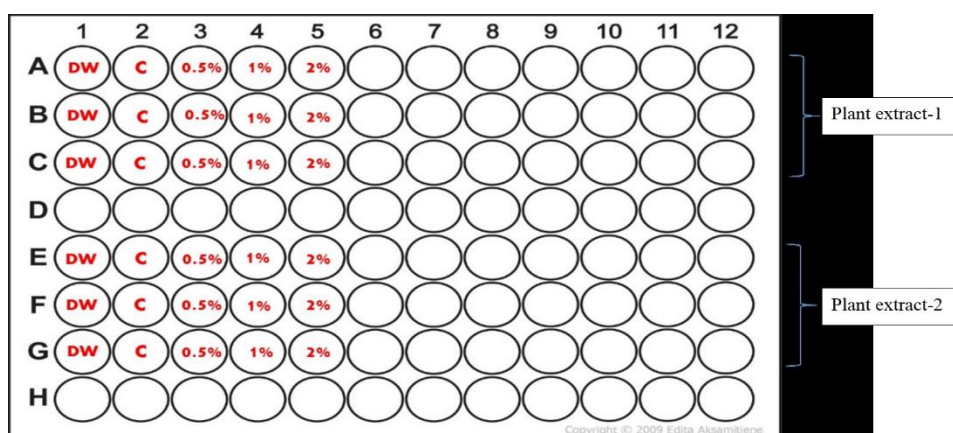


Fig.4: Figure showing 96-well format for Resazurin assay for plant extracts

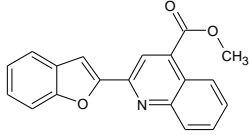
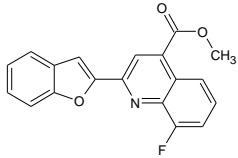
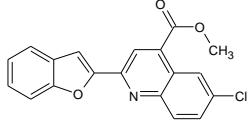
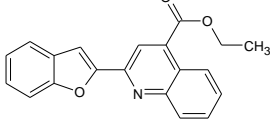
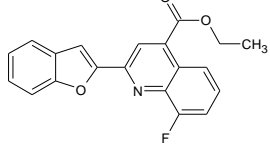
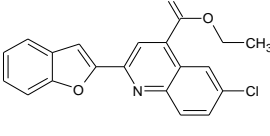
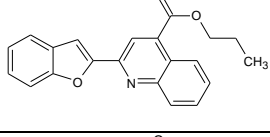
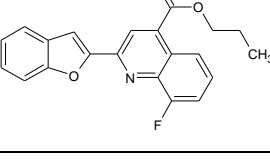
DW- Distilled water, C- Control

3.7. Screening of Synthetic Chemicals

3.7.1. Collection of synthetic chemicals

Synthetic chemicals of lactone based (64) were obtained from Kuvempu University, Shivamogga.

Table 3a. Chemical structure of synthetic chemicals (Batch-I)

Sl. No.	Structures	Code
1		4a
2		4b
3		4c
4		5a
5		5b
6		5c
7		6a
8		6b

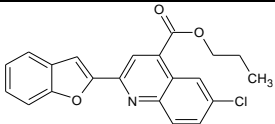
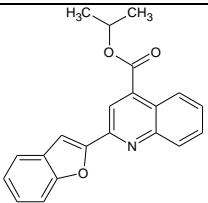
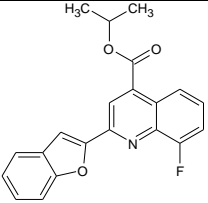
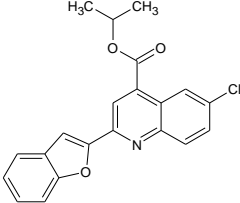
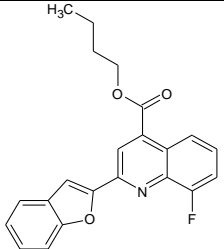
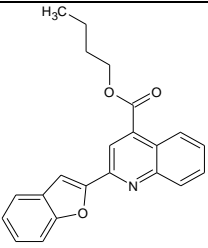
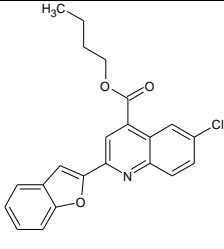
9		6c
10		7a
11		7b
12		7c
13		8a
14		8b
15		8c

Table 3b. Chemical structure of synthetic chemicals (Batch-II)

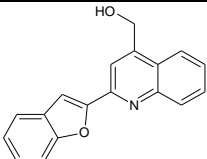
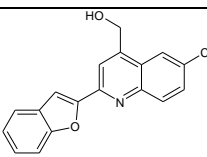
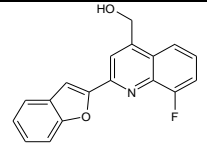
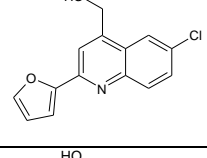
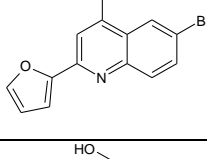
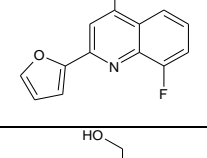
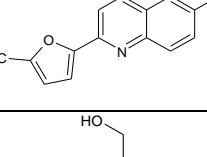
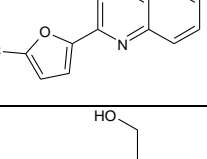
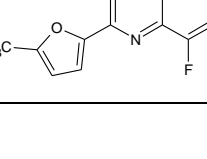
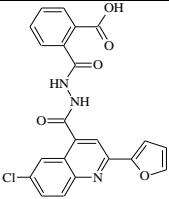
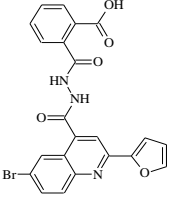
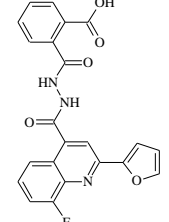
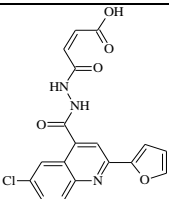
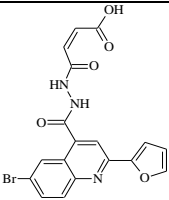
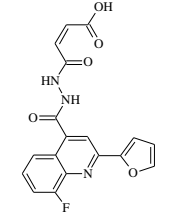
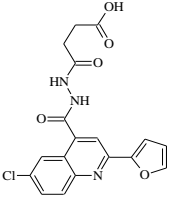
Sl. No.	Structures	Code
1		5a
2		5b
3		5c
4		10a
5		10b
6		10c
7		10d
8		10e
9		10f

Table 3c. Chemical structure of synthetic chemicals (Batch-III)

Sl. No.	Structures	Code
1		6a
2		6b
3		6c
4		7a
5		7b
6		7c
7		8a

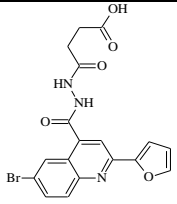
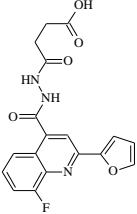
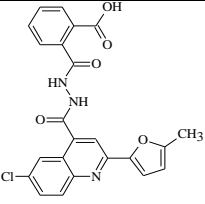
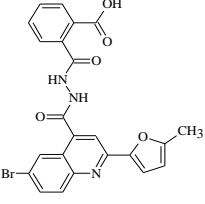
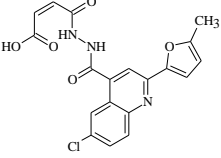
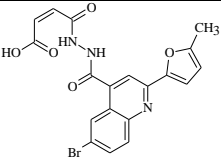
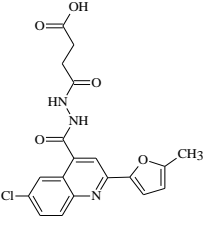
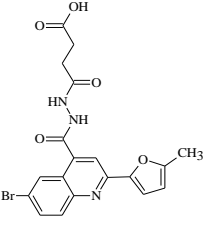
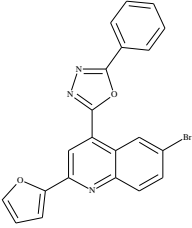
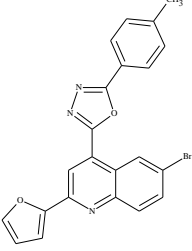
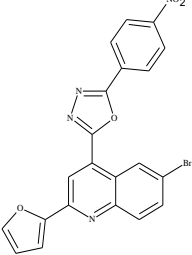
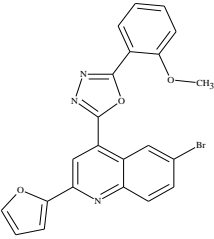
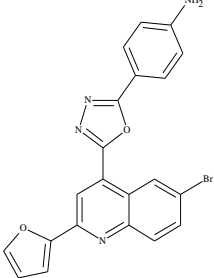
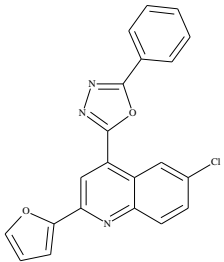
8		8b
9		8c
10		9a
11		9b
12		10a
13		10b
14		11a
15		11b

Table 3d. Chemical structure of synthetic chemicals (Batch-IV)

Sl. No.	Structures	Code
1		7a
2		7b
3		7c
4		7d
5		7e
6		8a

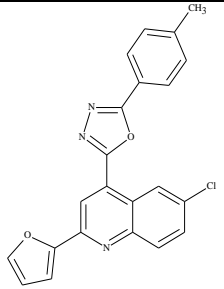
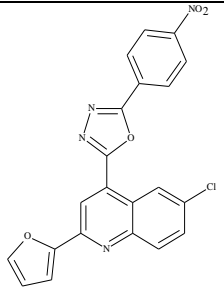
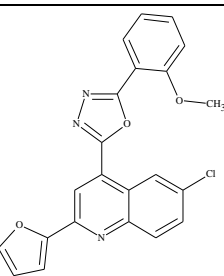
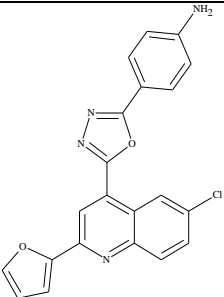
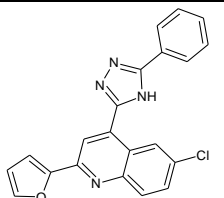
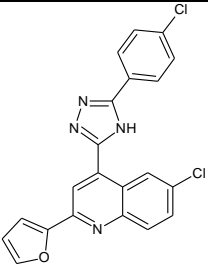
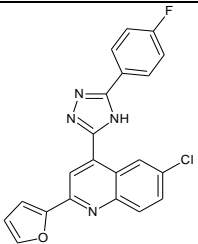
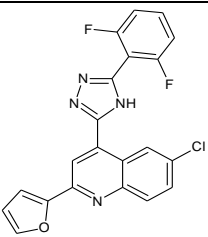
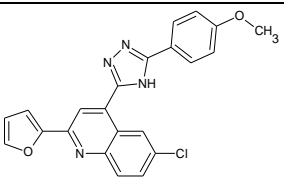
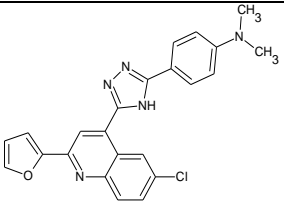
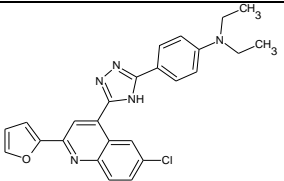
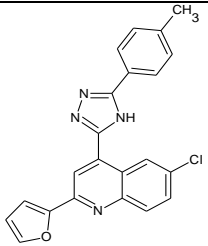
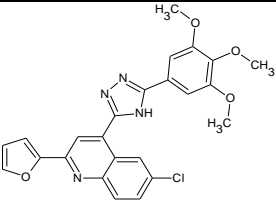
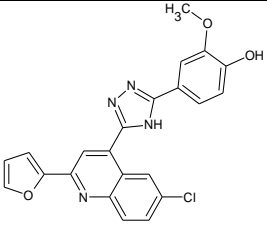
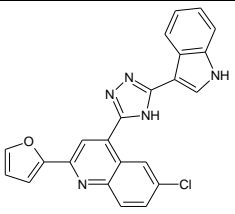
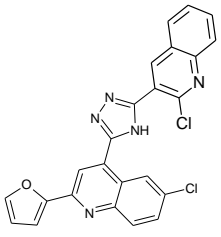
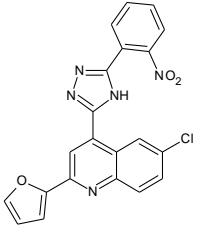
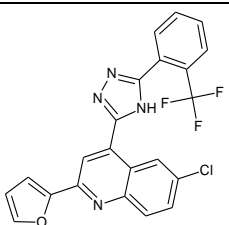
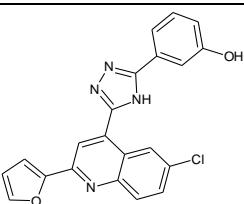
7		8b
8		8c
9		8d
10		8e

Table 3e. Chemical structure of synthetic chemicals (Batch-V)

Sl. No.	Structures	Code
1		7a

2		7b
3		7c
4		7d
5		7e
6		7f
7		7g
8		7h

9		7i
10		7j
11		7k
12		7l
13		7m
14		7n
15		7o

3.7.2. Dilution of synthetic chemicals

Each synthetic chemical was diluted at different concentrations such as 1, 10 and 100 ppm in sterile distilled water.

3.7.3. Screening of synthetic chemicals against *A. flavus* and *R. bataticola* by using Resazurin assay

3.7.3.1. Preparation of Resazurin solution

0.02 per cent of Resazurin solution was prepared by dissolving 2 mg in 10 ml of sterile distilled water. The solution was vortexed and dissolved to make homogenous solution.

3.7.3.2. Preparation of the plates

Plates were prepared under aseptic conditions. The plates were prepared in triplicate. A sterile 96-well plate was labelled. 180 μ l of PDB, 20 μ l of *A. flavus*/*R. bataticola* culture was added into all the wells and diluted synthetic chemicals were added into the labelled wells accordingly in 3 different concentrations (1, 10 and 100 ppm). *i.e.*, 0.5 μ l, 5 μ l and 50 μ l for 200 μ l. Controls were maintained without synthetic chemicals. Sealed with parafilm and incubated at 28 ± 2 °C for 5 days. After 5 days of incubation 20 μ l of 0.02 per cent Resazurin solution was added into all wells and incubated for 30 min. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value (Bonnier *et al.*, 2015).

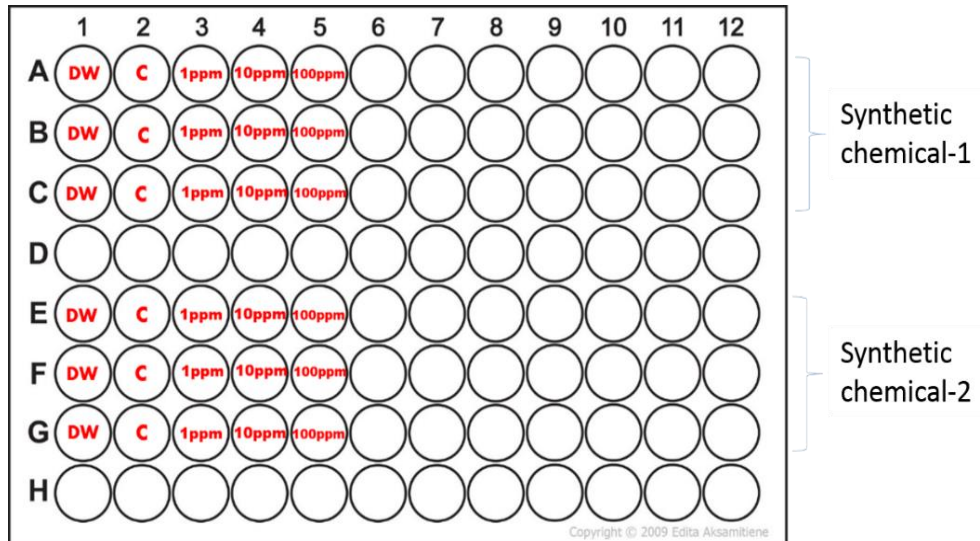


Fig 5. Figure showing 96-well format for Resazurin assay for synthetic chemicals
DW- Distilled water, C- Control

IV. EXPERIMENTAL RESULTS

The present investigation was envisaged to study the effect of 12 essential oils, 24 plant extracts and 64 chemical entities against *A. flavus* and *R. bataticola*. Results obtained from the present investigation are furnished under the following headings.

4.1. Screening of essential oils against *A. flavus* and *R. bataticola*

4.2 Screening of plant extracts against *A. flavus* and *R. bataticola*

4.3. Screening of synthetic chemicals against *A. flavus* and *R. bataticola*

4.1. Screening of essential oils against *A. flavus* and *R. bataticola*

4.1.1. Screening of essential oils against *A. flavus* and *R. bataticola* by using Micro titer plate

4.1.1.1. Micro plate readings of *A. flavus* growth at different time interval for different essential oils at OD 595 nm

Orange oil:

The orange oil was tested for the growth inhibition of fungal mycelia by incubating the fungal spores at orange oil concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent), time hour of incubation a maximum of 7 days (0, 24, 48, 72, 96, 120, 144 and 168 hr), the absorbance reading was measured at the OD 595 nm for the above time interval and the data recorded is presented as below.

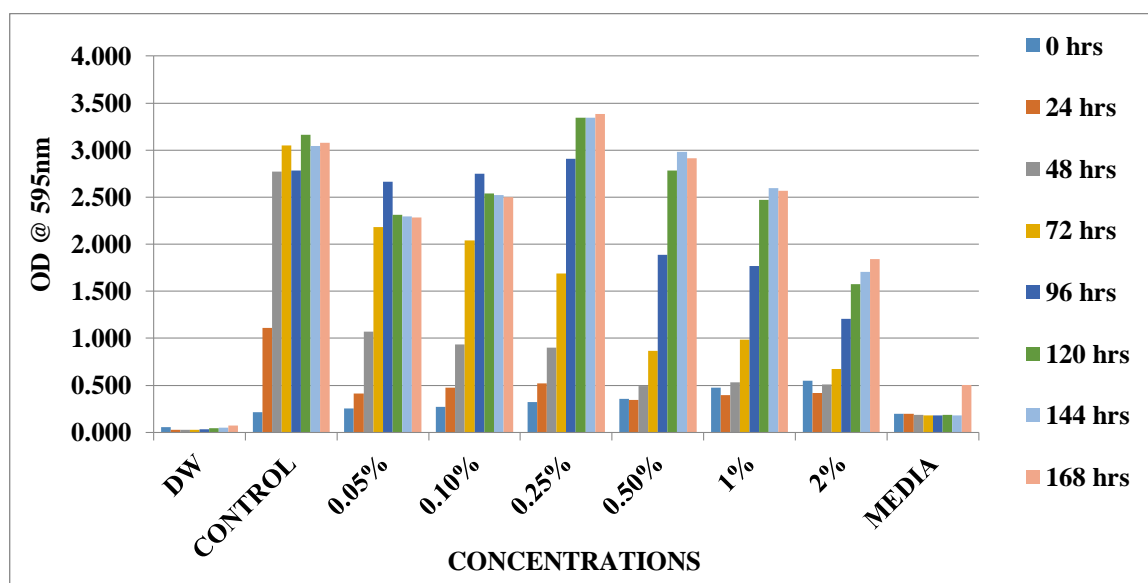


Fig 6. *In vitro* efficacy of orange oil against *A. flavus*

The orange oil inhibited the *A. flavus* growth at lower concentrations (0.05 % to 0.5 %) observed was nil, at 1 per cent observed was 20 per cent and at 2 per cent found was 40 per cent. Hence this oil is not suitable for the control of *A. flavus*.

Peppermint oil:

The peppermint oil concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) was tested for the inhibition of growth of fungus and the data recorded is presented as below.

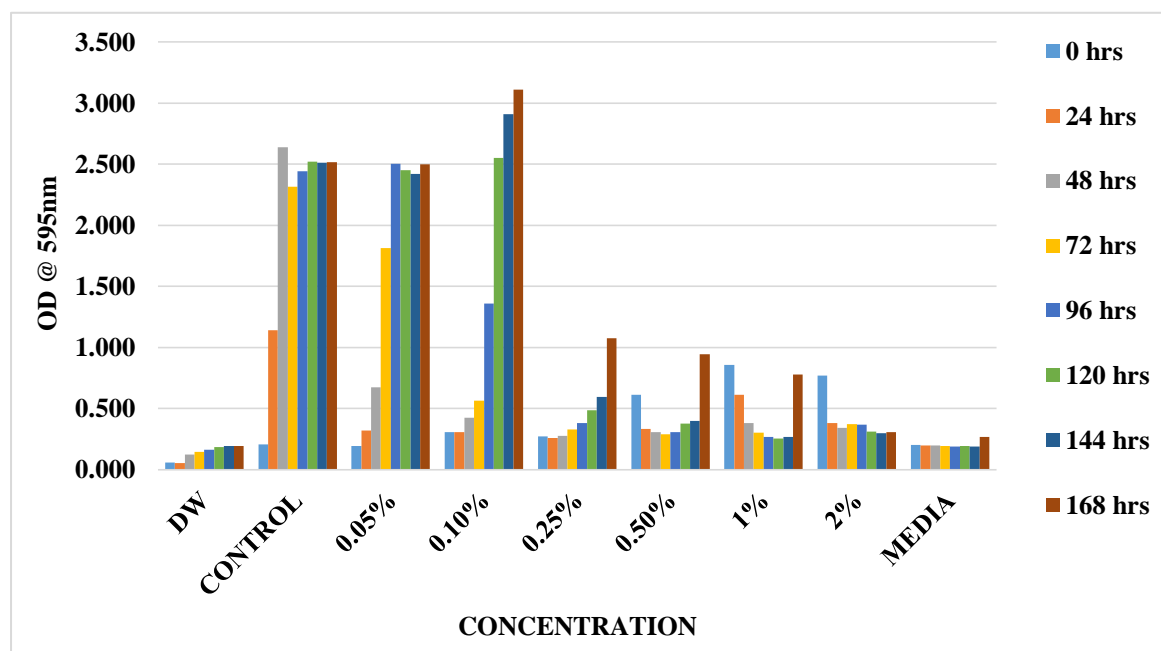


Fig 7. *In vitro* efficacy of peppermint against *A. flavus*

The peppermint oil inhibited the *A. flavus* growth at lower concentrations (0.05 % to 0.25 %) found was insignificant, at 0.5 per cent observed was 40 per cent, at 1 per cent observed was 70 per cent and at 2 per cent found was 80 per cent.

Citronella oil:

The citronella oil was tested for the evaluation of efficacy against fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

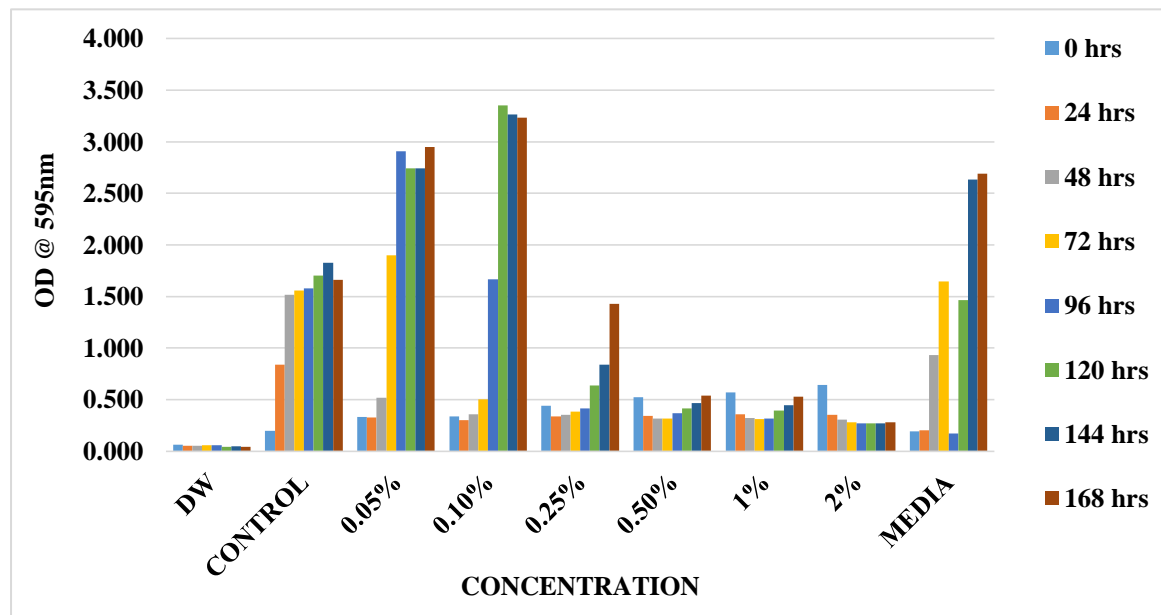


Fig 8. *In vitro* efficacy of citronella oil against *A. flavus*

The citronella oil inhibited the *A. flavus* growth at lower concentrations (0.05 % to 0.25 %) found was insignificant, at 0.5 per cent observed was 60 per cent, at 1 per cent observed was 65 per cent and at 2 per cent found was 80 per cent.

Methyl eugenol:

The methyl eugenol was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

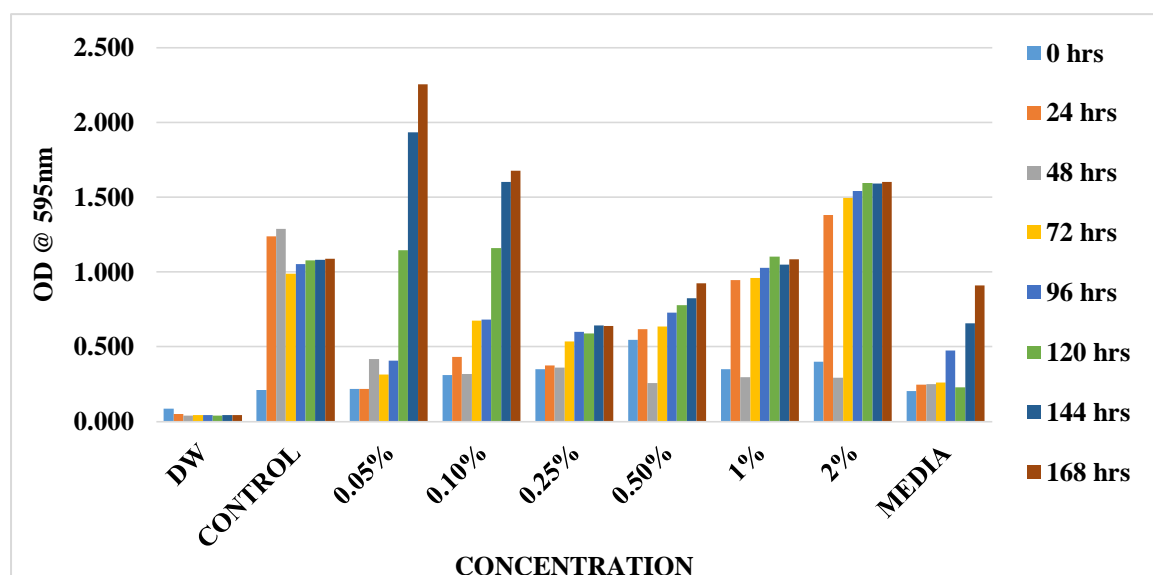


Fig 9. In vitro efficacy of methyl eugenol against *A. flavus*

The methyl eugenol inhibited the *A. flavus* growth at lower concentrations (0.05 % and 0.1 %) observed was insignificant, at 0.25 per cent found was 50 per cent and as the concentration of methyl eugenol increases the wells appeared cloudy and whitish, hence it affected the spectral readings.

Eugenol:

The eugenol was tested for the growth inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent), time hour of incubation a maximum of 7 days (0, 24, 48, 72, 96, 120, 144 and 168 hr), the absorbance reading was measured at the OD 595 nm for the above time interval and the data recorded is presented as below.

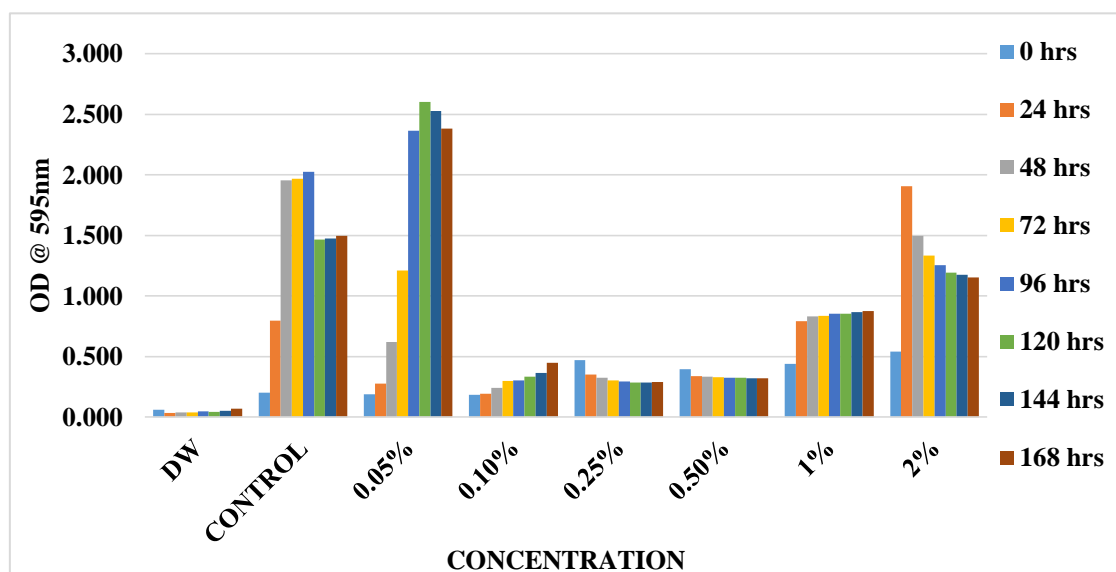


Fig 10. *In vitro* efficacy of eugenol against *A. flavus*

The eugenol inhibited the *A. flavus* growth at 0.25 per cent obtained was 80 per cent and as the concentration of eugenol increases the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that the Eugenol has inhibited *A. flavus* growth completely at 0.5, 1 and 2 per cent (Plate 1).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the eugenol inhibits the growth of *A. flavus* from 0.5 per cent onwards.

Ajowain seed oil:

The ajowain seed oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

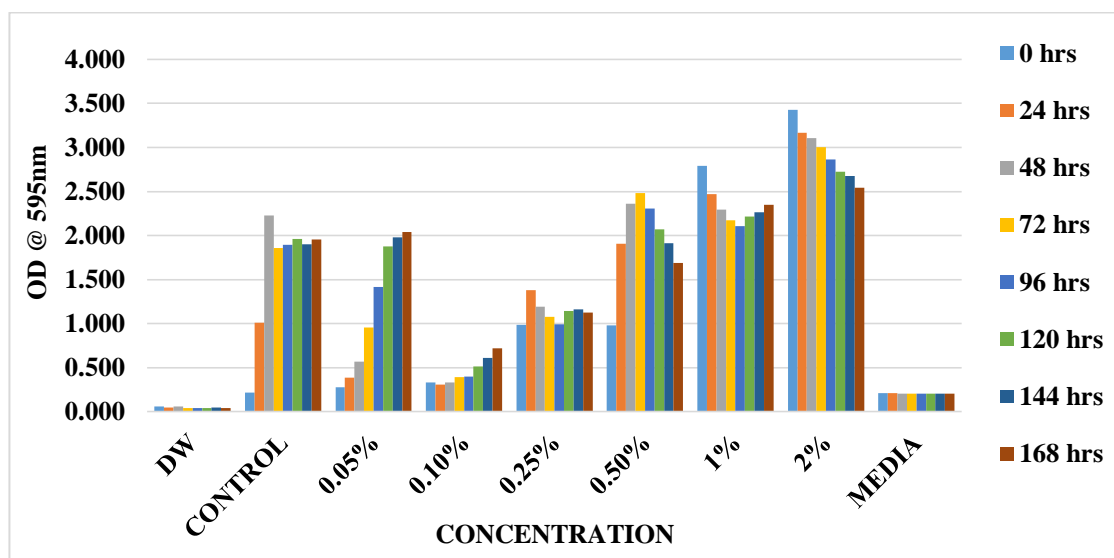


Fig 11. *In vitro* efficacy of ajowain seed oil against *A. flavus*

The ajowain seed oil inhibited the *A. flavus* growth at 0.1 per cent obtained was 65 per cent and as the concentration of ajowain seed oil increases the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100 per cent inhibition at 0.5, 1 and 2 per cent (Plate 2).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the ajowain seed oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

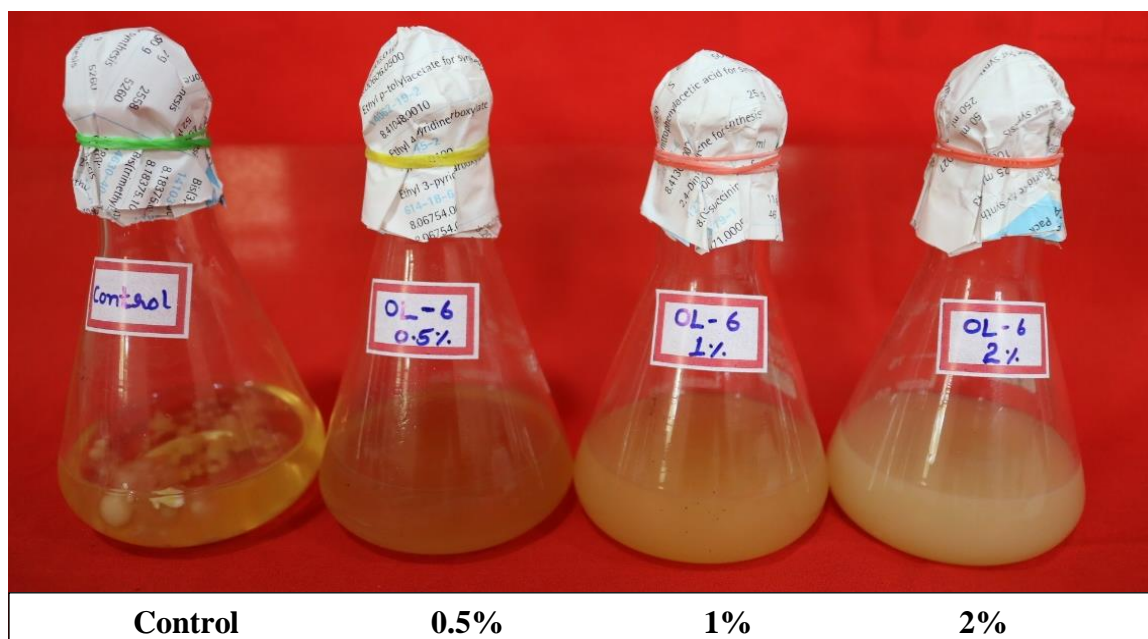


Plate 1. *In vitro* efficacy of Eugenol against *A. flavus* in PDB

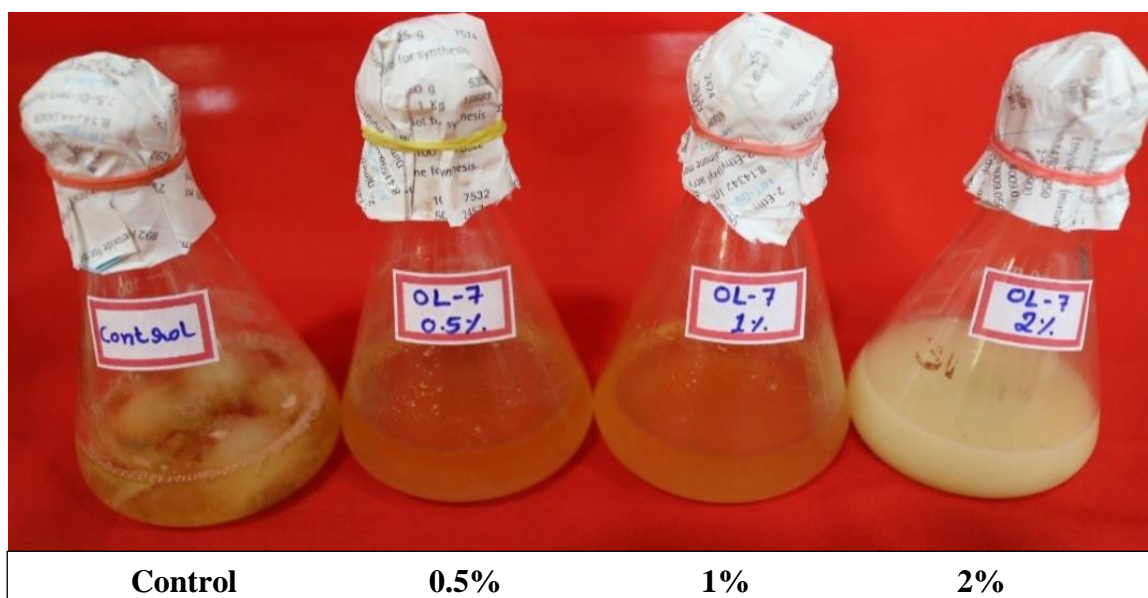


Plate 2. *In vitro* efficacy of Ajowain seed oil against *A. flavus* in PDB

Cumin seed oil:

The cumin seed oil was tested for the evaluation of efficacy against fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

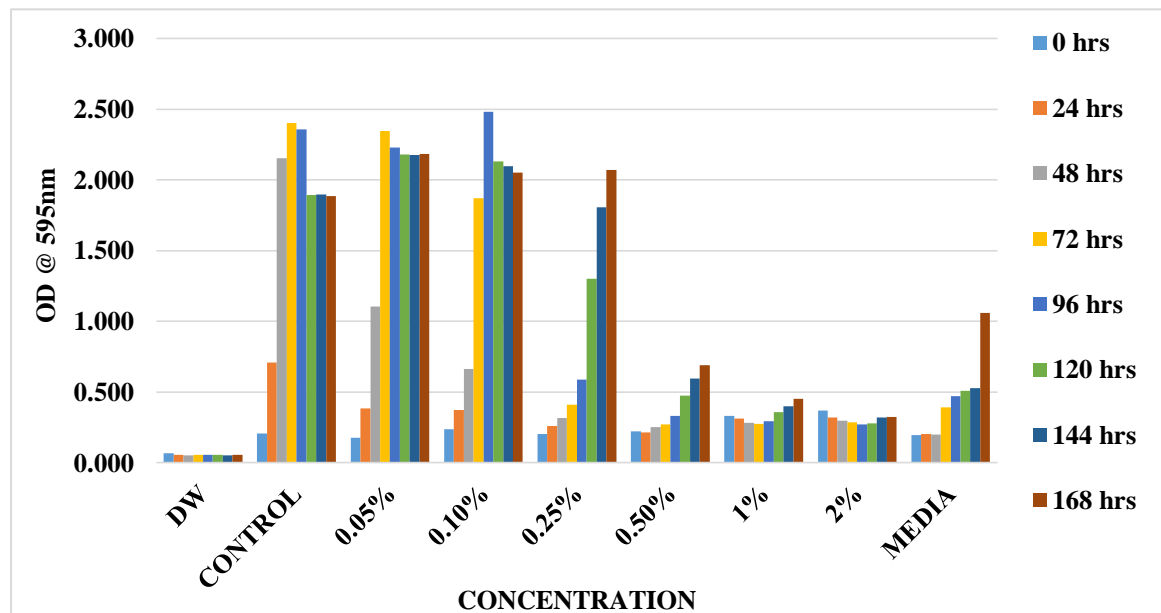


Fig 12. *In vitro* efficacy of cumin seed oil against *A. flavus*

The cumin seed oil inhibited the *A. flavus* growth at lower concentrations (0.05 % to 0.25 %) found was insignificant, at 0.5 per cent found was 60 per cent, at 1 per cent found was 70 per cent and at 2 per cent obtained was 80 per cent.

Rose oil:

The rose oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

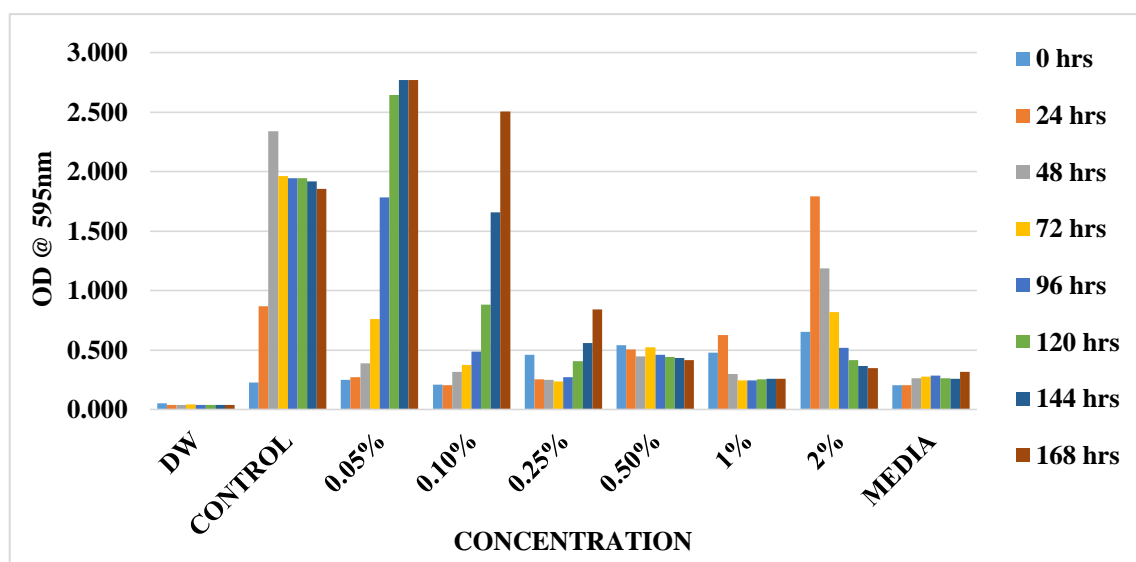


Fig 13. *In vitro* efficacy of rose oil against *A. flavus*

The rose oil inhibited the *A. flavus* growth at lower concentrations (0.05 % to 0.25 %) observed was insignificant, at 0.5 per cent obtained was 80 per cent, at 1 per cent obtained was 90 per cent and at 2 per cent the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100 per cent inhibition at 2 per cent (Plate 3).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the rose oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

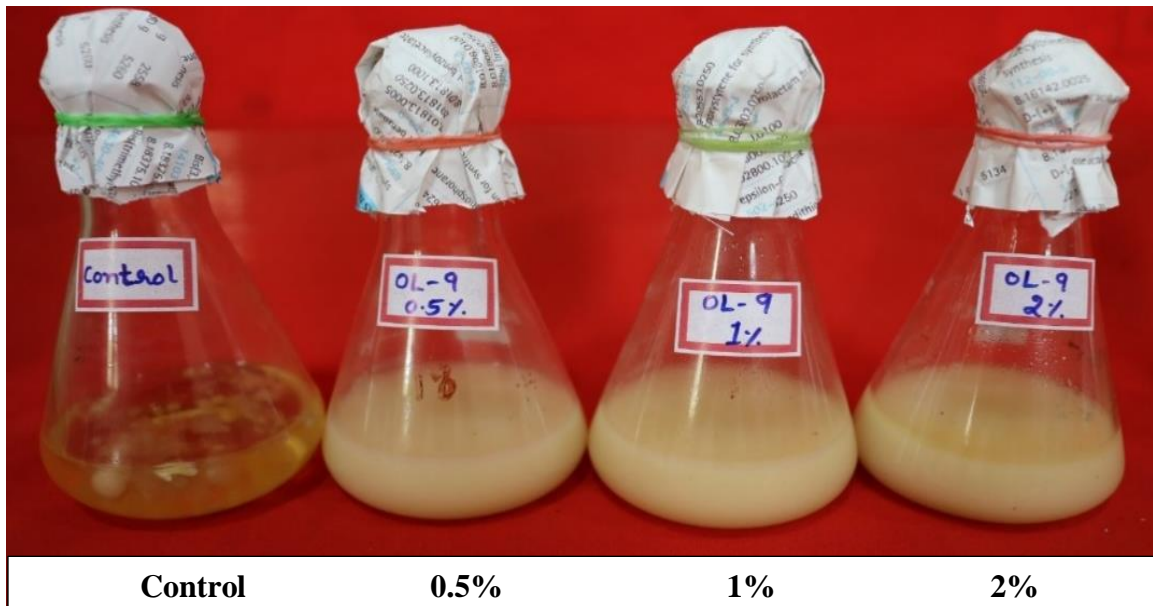


Plate 3. *In vitro* efficacy of Rose oil against *A. flavus* in PDB

Lavender oil:

The lavender oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

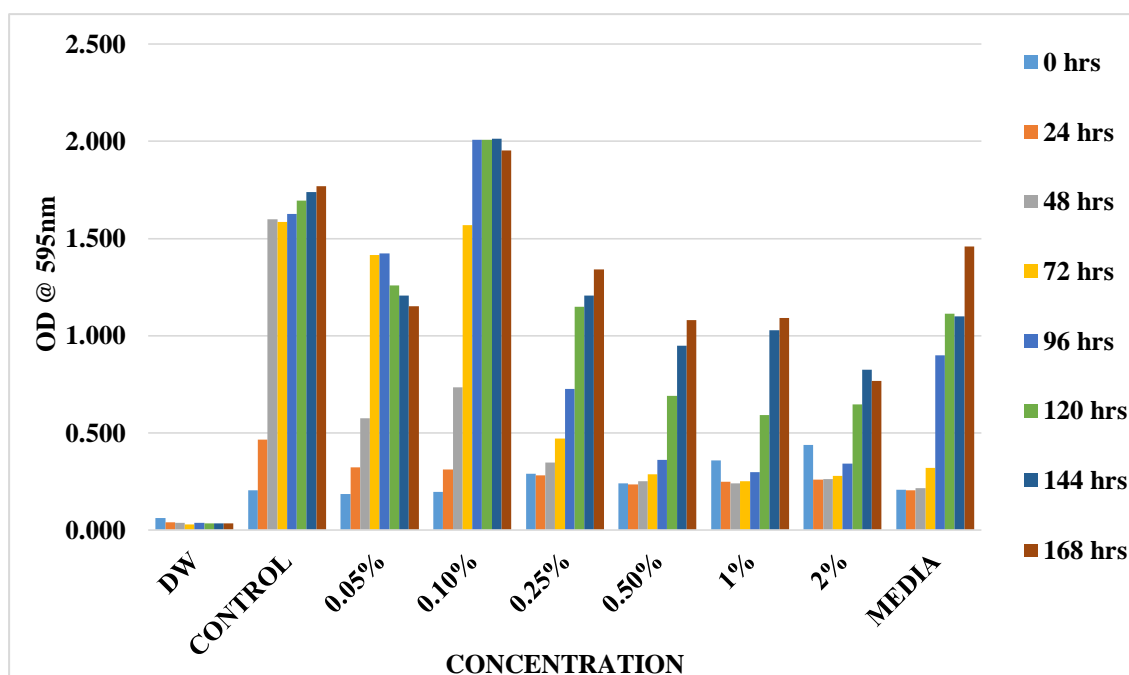


Fig 14. *In vitro* efficacy of lavender oil against *A. flavus*

The lavender oil inhibited the *A. flavus* growth at lower concentrations (0.05 % to 0.5 %) observed was insignificant, at 1 per cent found was 40 per cent and at 2 per cent obtained was 65 per cent.

Clove oil:

The clove oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

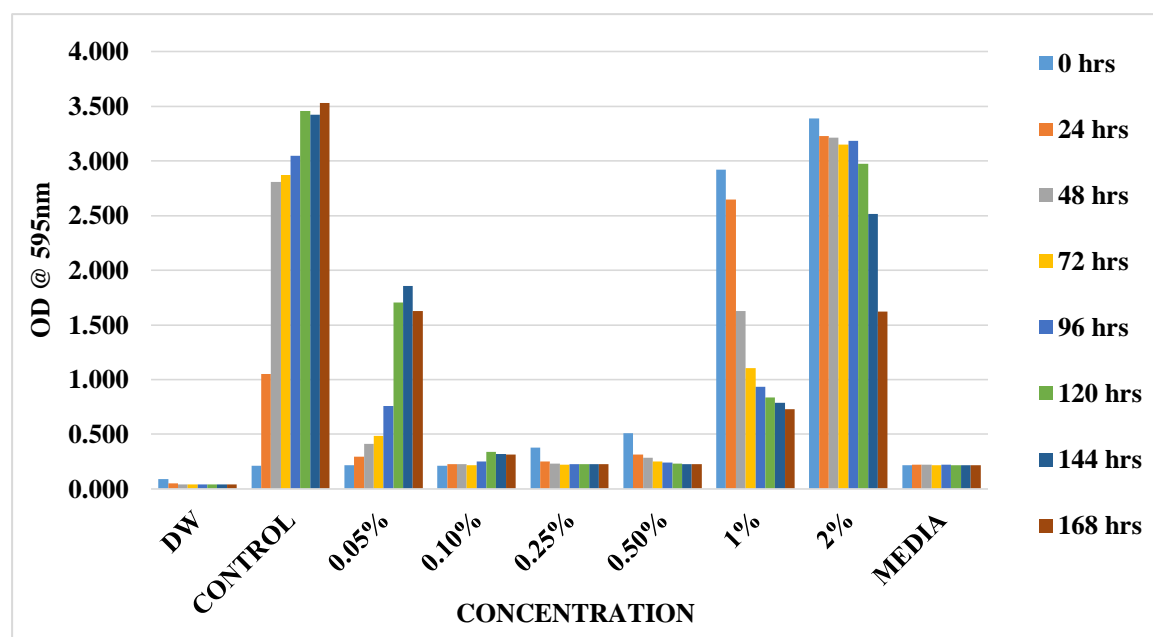


Fig 15. *In vitro* efficacy of clove oil against *A. flavus*

The clove oil inhibited the *A. flavus* growth at 0.1 and 0.25 per cent obtained was 92 and 94 per cent respectively, but at 0.5, 1 and 2 per cent the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100 per cent inhibition (Plate 4).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the clove oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

Cinnamon oil:

The cinnamon oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

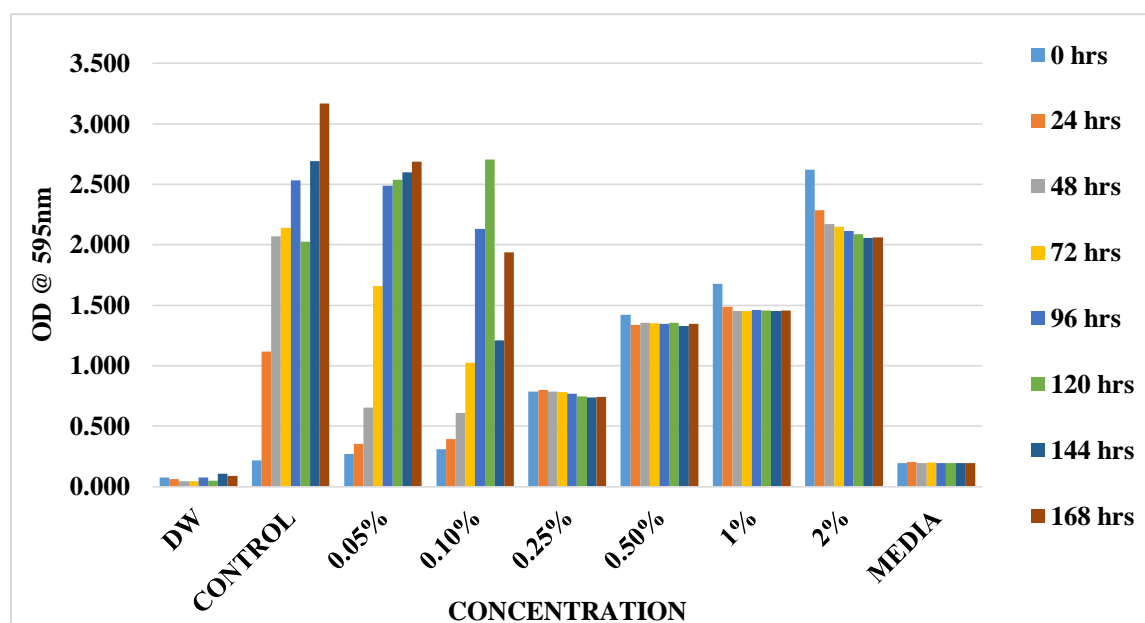


Fig 16. *In vitro* efficacy of cinnamon oil against *A. flavus*

The cinnamon oil inhibited the *A. flavus* growth at 0.1 and 0.25 per cent obtained was 61 and 77 per cent respectively. As the concentration of cinnamon oil increases the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100per cent inhibition (Plate 5).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the cinnamon oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

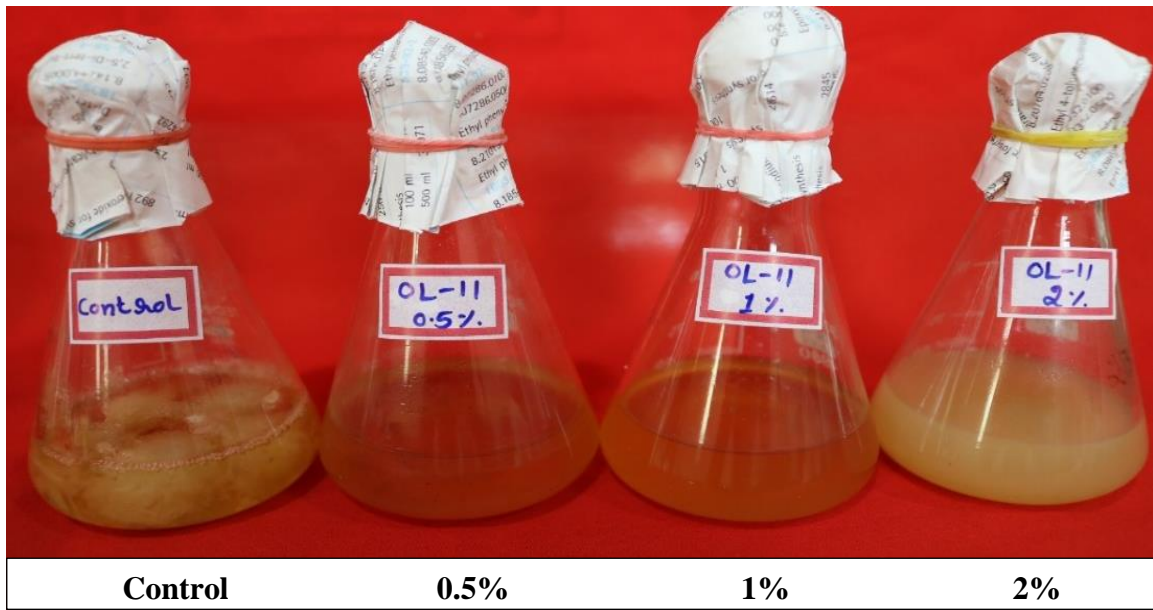


Plate 4. *In vitro* efficacy of Clove oil against *A. flavus* in PDB

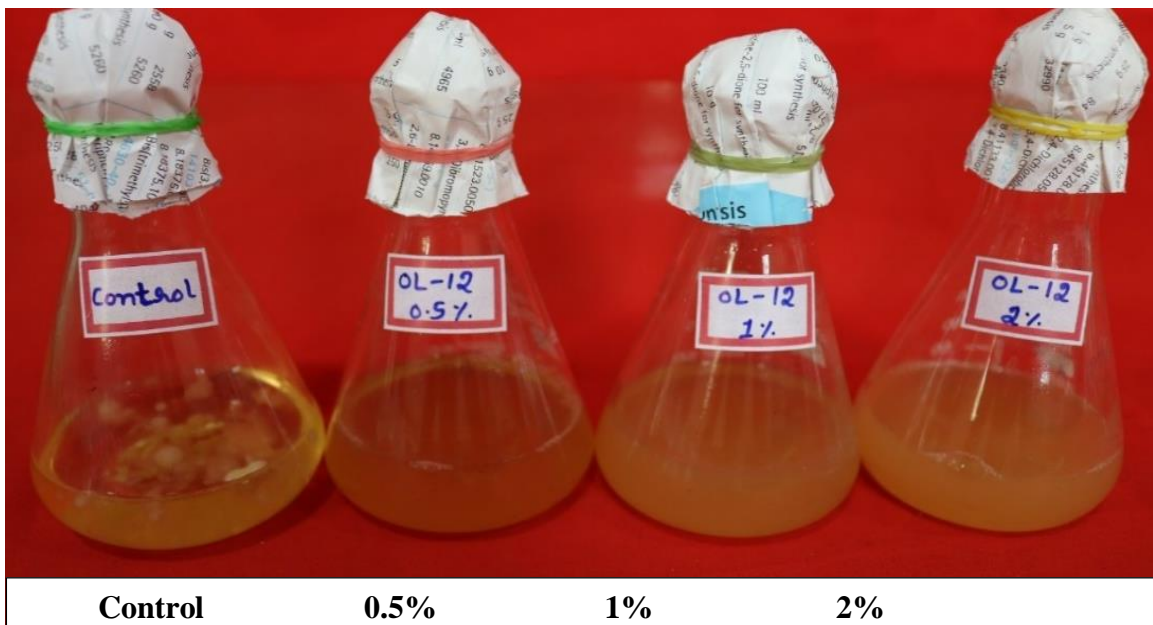


Plate 5. *In vitro* efficacy of Cinnamon oil against *A. flavus* in PDB

Eucalyptus oil:

The eucalyptus oil was tested for the evaluation of efficacy against fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

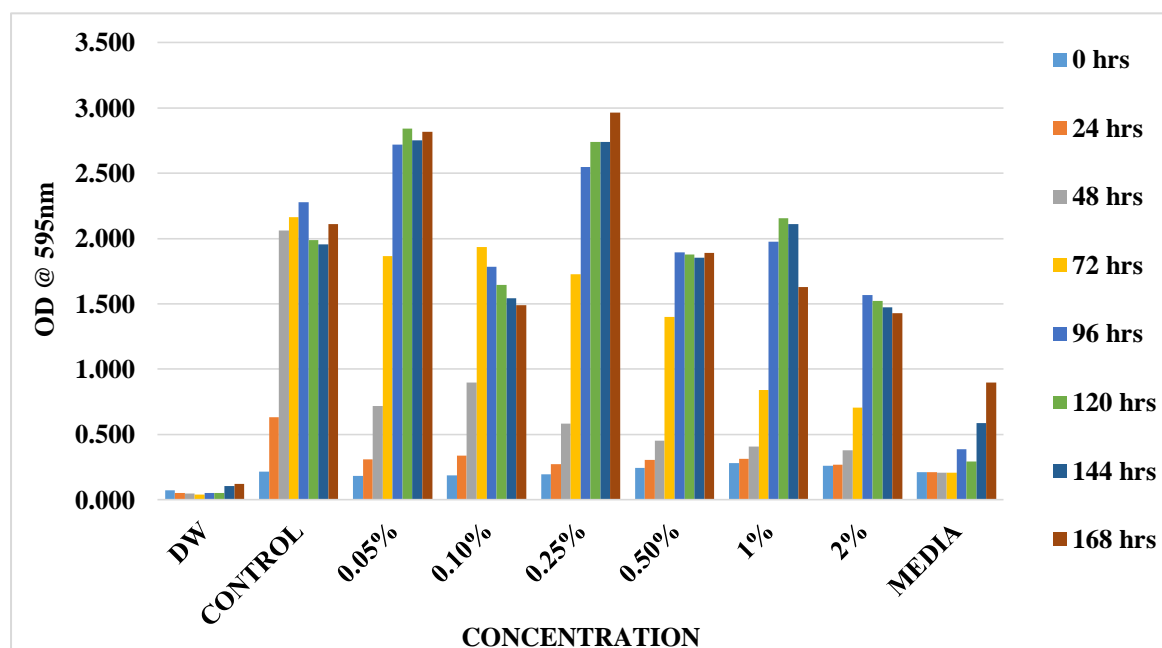


Fig 17. *In vitro* efficacy of eucalyptus oil against *A. flavus*

The eucalyptus essential oil inhibited the *A. flavus* growth at lower concentrations (0.05 % to 1 %) observed was insignificant, at 2 per cent found was 32 per cent. Hence this oil is not suitable for the control of *A. flavus*. Hence this oil is not suitable for the control of *A. flavus*.

4.1.1.2. Micro plate readings of *R. bataticola* growth at different time interval for different essential oils at OD 595 nm

Orange oil:

The orange oil was tested for the growth inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent), time hour of incubation a maximum of 7 days (0, 24, 48, 72, 96, 120, 144 and 168 hr), the absorbance reading was measured at the OD 595 nm for the above time interval and the data recorded is presented as below.

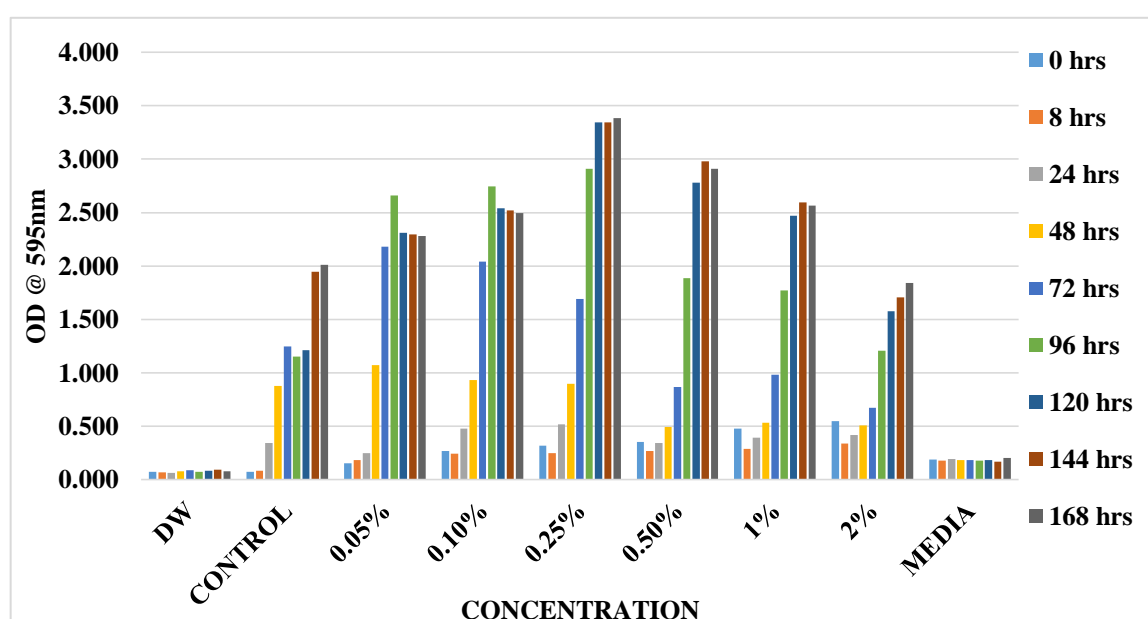


Fig 18. *In vitro* efficacy of orange oil against *R. bataticola*

The orange oil inhibited the *R. bataticola* growth at lower concentrations (0.05 % to 1 %) observed was insignificant and at 2 per cent found was 9 per cent. Hence this oil is not suitable for the control of *R. bataticola*.

Peppermint oil:

The peppermint oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

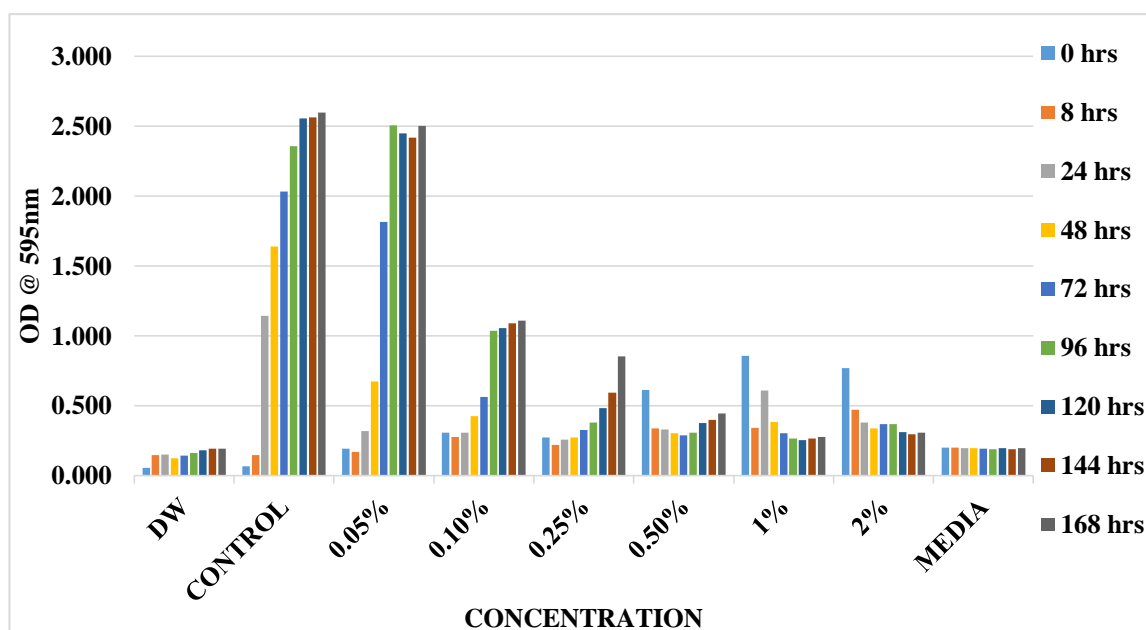


Fig 19. *In vitro* efficacy of peppermint oil against *R. bataticola*

The peppermint oil inhibited the *R. bataticola* growth at lower concentrations (0.05 % to 0.25 %) observed was insignificant, at 0.5, 1 and 2 per cent found was 70, 80 and 88 per cent respectively.

Citronella oil:

The citronella oil was tested for the evaluation of efficacy against fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

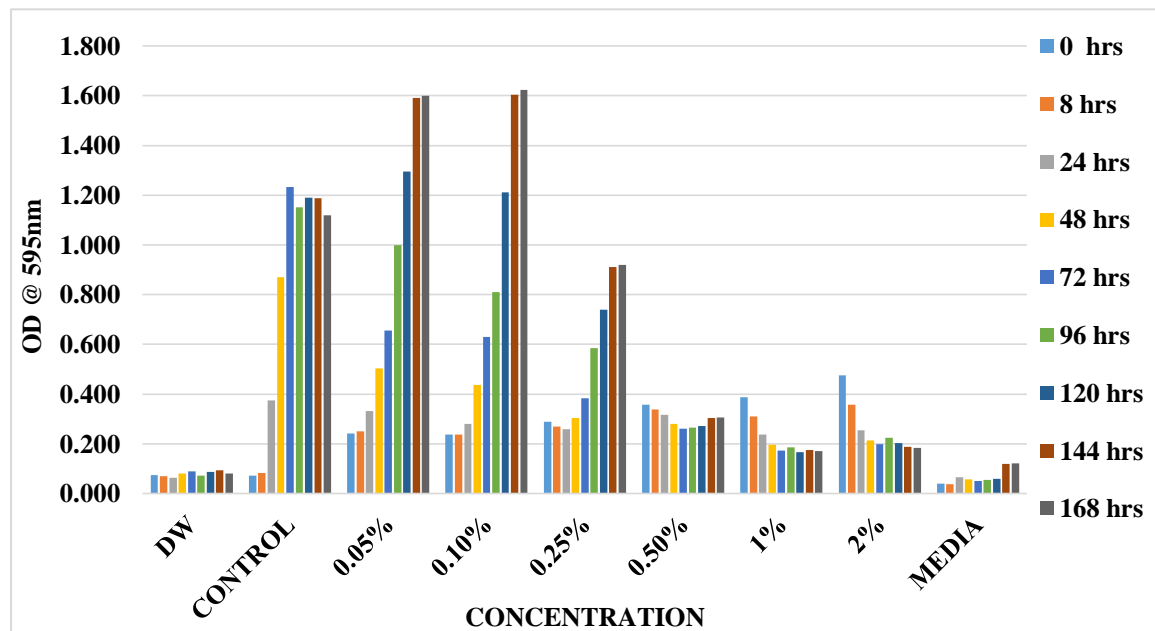


Fig 20. *In vitro* efficacy of citronella oil against *R. bataticola*

The citronella oil inhibited the *R. bataticola* growth at lower concentrations (0.05 % to 0.25 %) observed was insignificant, at 0.5, 1 and 2 per cent found was 70, 79 and 83 per cent respectively.

Methyl eugenol:

The methyl eugenol was tested for the controlling fungal mycelia by incubating the fungal inoculum at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

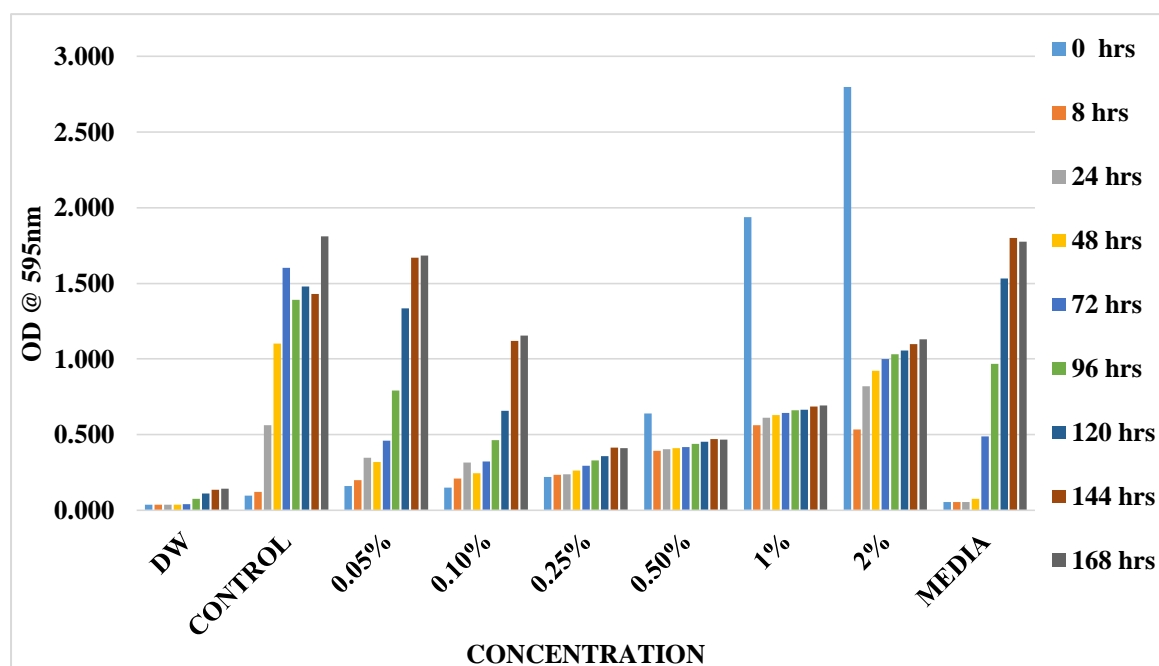


Fig 21. *In vitro* efficacy of methyl eugenol against *R. bataticola*

The methyl eugenol inhibited the *R. bataticola* growth at lower concentrations observed was insignificant, at 0.25 per cent found was 77 per cent and as the concentration of methyl eugenol increases the wells appeared cloudy and whitish, hence it affected the spectral readings.

Eugenol:

The eugenol was tested for the growth inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent), time hour of incubation a maximum of 7 days (0, 24, 48, 72, 96, 120, 144 and 168 hr), the absorbance reading was measured at the OD 595 nm for the above time interval and the data recorded is presented as below.

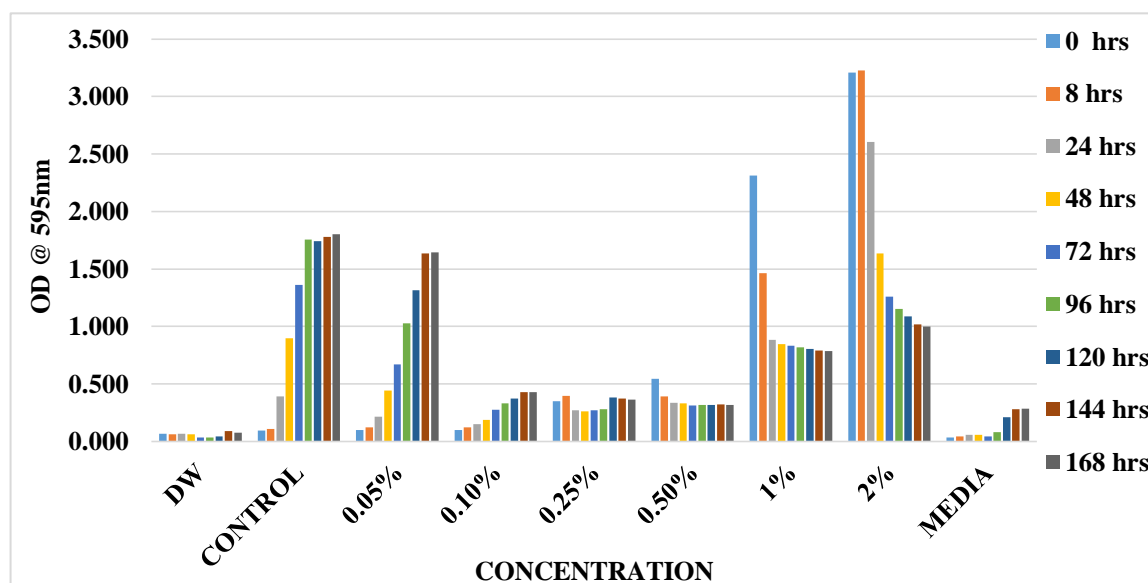


Fig 22. In vitro efficacy of eugenol against *R. bataticola*

The eugenol inhibited the *R. bataticola* growth at 0.25 per cent found was 81 per cent and as the concentration of eugenol increases the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that eugenol has inhibited *R. bataticola* growth completely at 0.5, 1 and 2 per cent (Plate 6).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the eugenol essential oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

Ajowain seed oil:

The ajowain seed oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

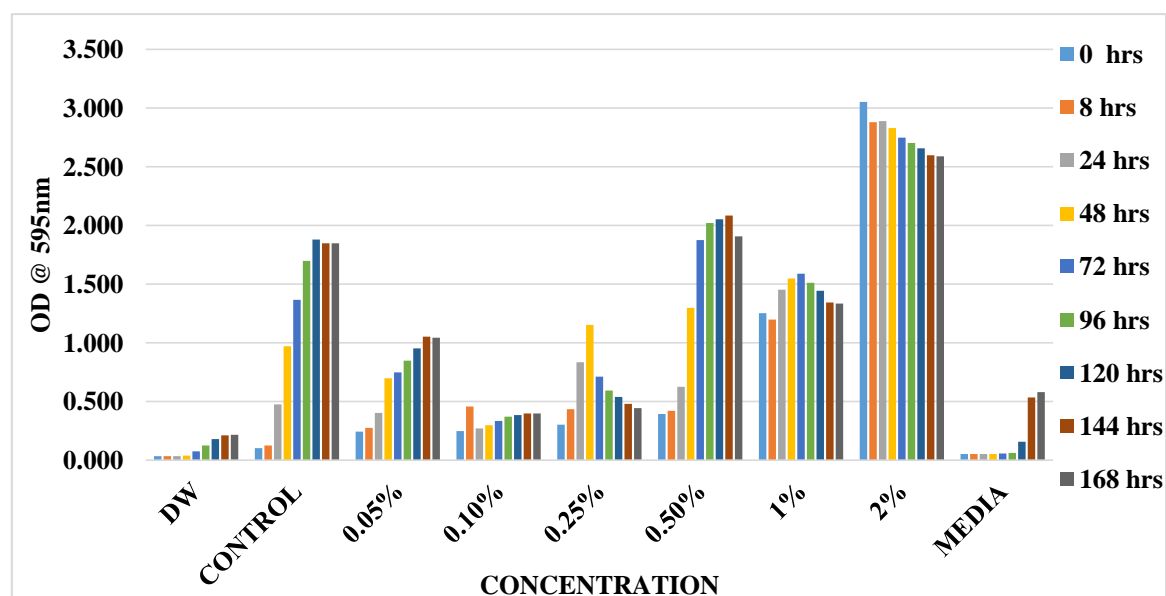


Fig 23. *In vitro* efficacy of ajowain seed oil against *R. bataticola*

The ajowain seed oil inhibited the *R. bataticola* growth at 0.1 per cent obtained was 79 per cent and as the concentration of ajowain seed oil increases the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100 per cent inhibition at 0.5, 1 and 2 per cent (Plate 7).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the ajowain seed oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

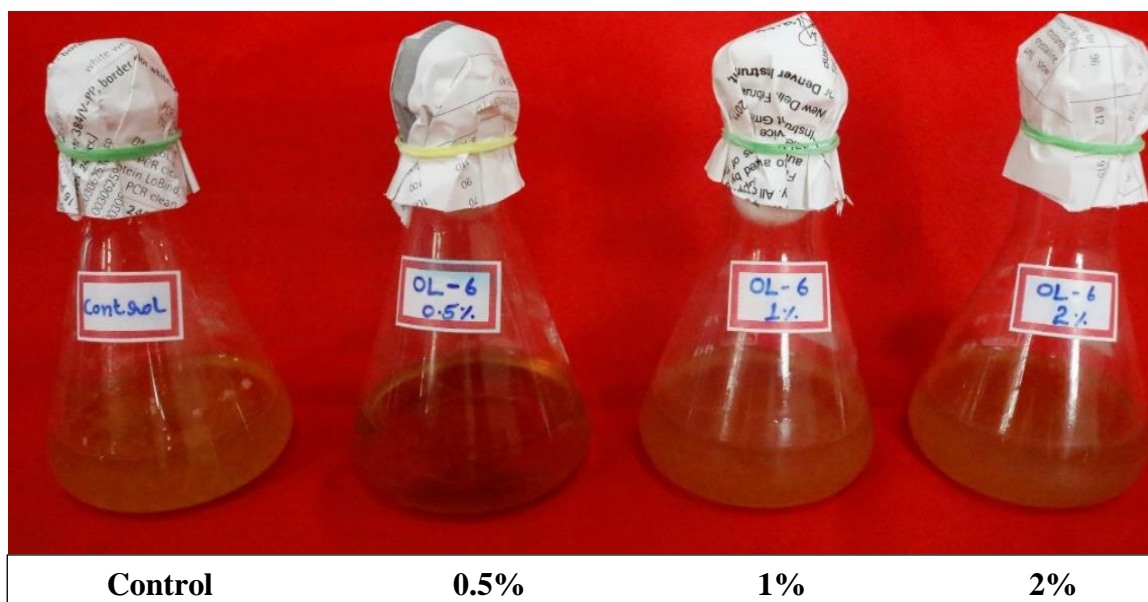


Plate 6. *In vitro* efficacy of Eugenol against *R. bataticola* in PDB

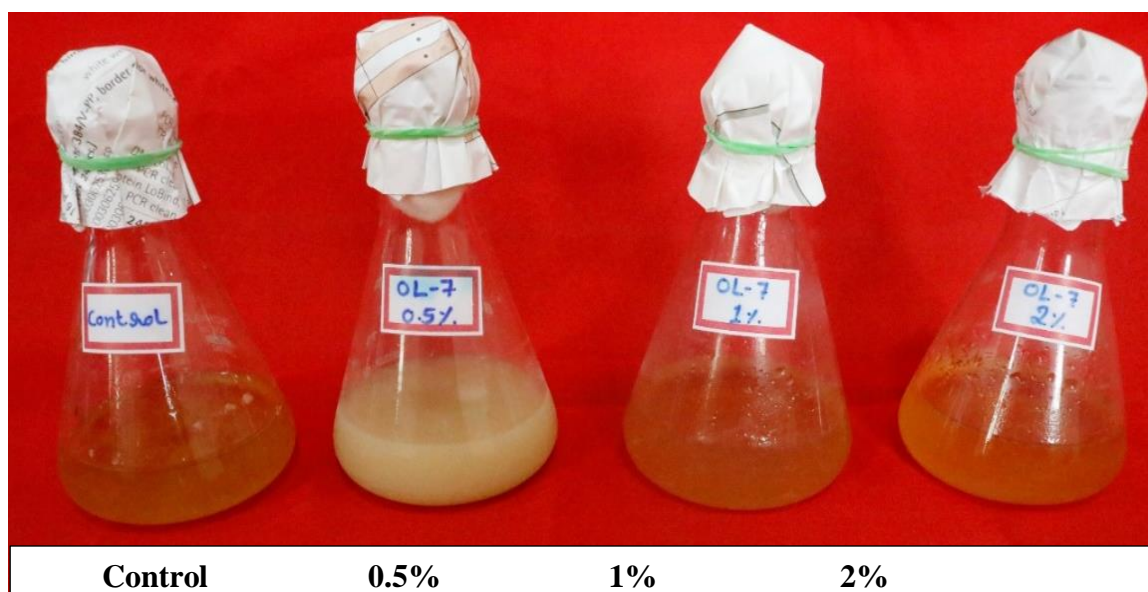


Plate 7. *In vitro* efficacy of Ajowain seed oil against *R. bataticola* in PDB

Cumin seed oil:

The cumin seed oil was tested for the evaluation of efficacy against fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

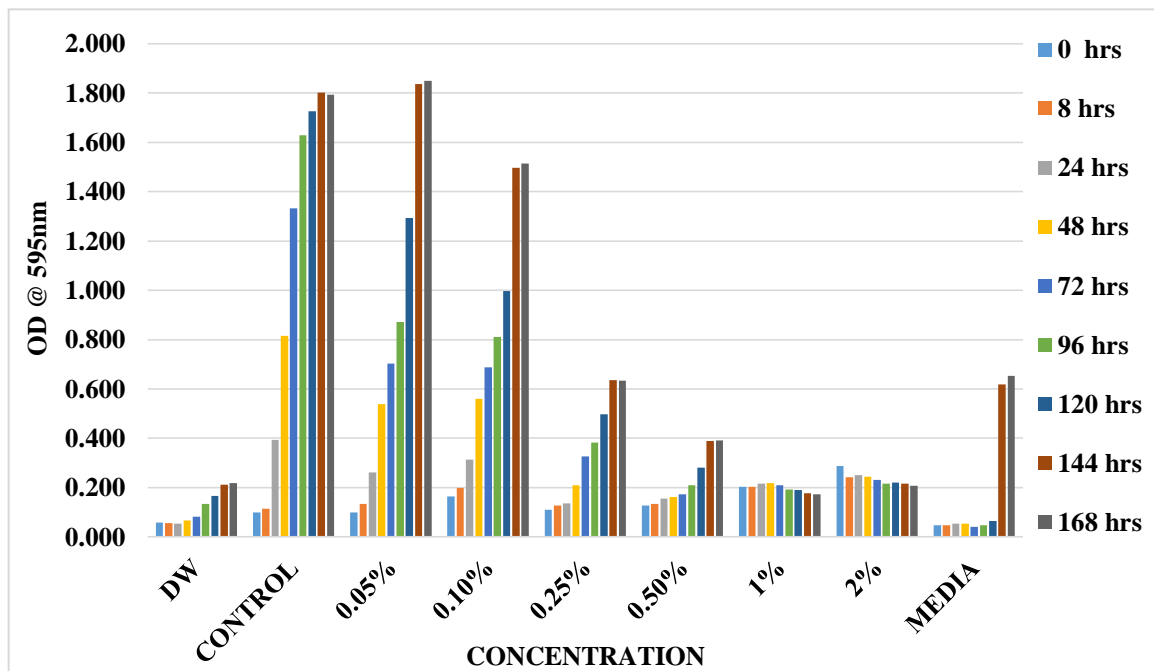


Fig 24. *In vitro* efficacy of cumin seed oil against *R. bataticola*

The cumin seed oil inhibited the *R. bataticola* growth at lower concentrations (0.05 % to 0.25 %) observed was insignificant, at 0.5, 1 and 2 per cent obtained was 80, 85 and 89 per cent respectively.

Rose oil:

The rose oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

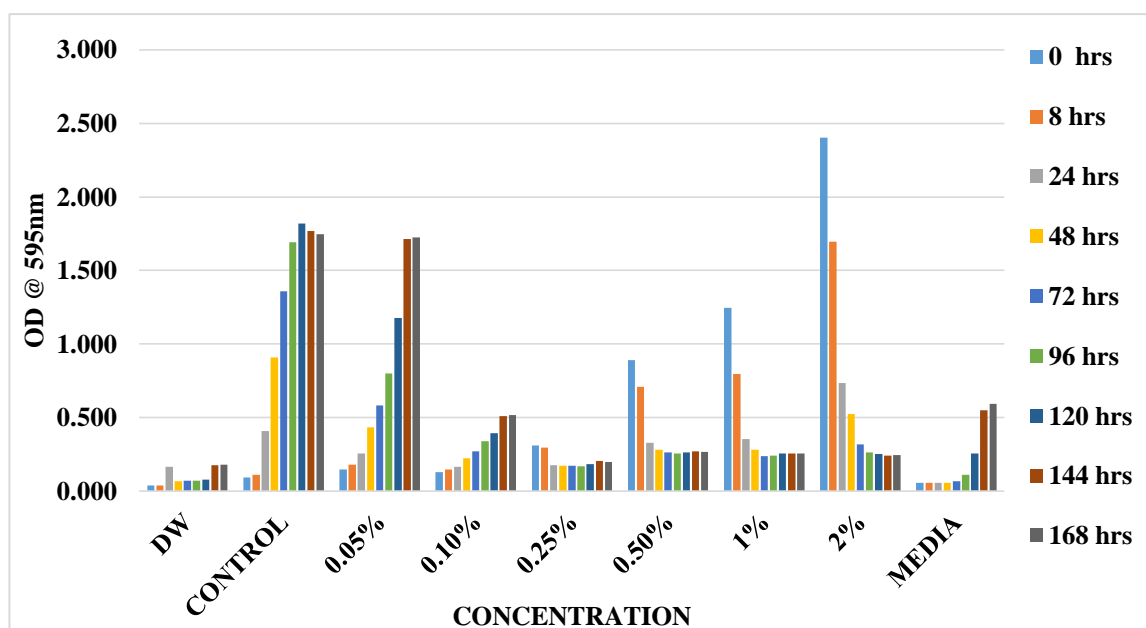


Fig 25. *In vitro* efficacy of rose oil against *R. bataticola*

The rose oil inhibited the *R. bataticola* growth at 0.25 per cent obtained was 89 per cent and at 0.5, 1 and 2 per cent the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100 per cent inhibition at higher concentrations (Plate 8).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the rose oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

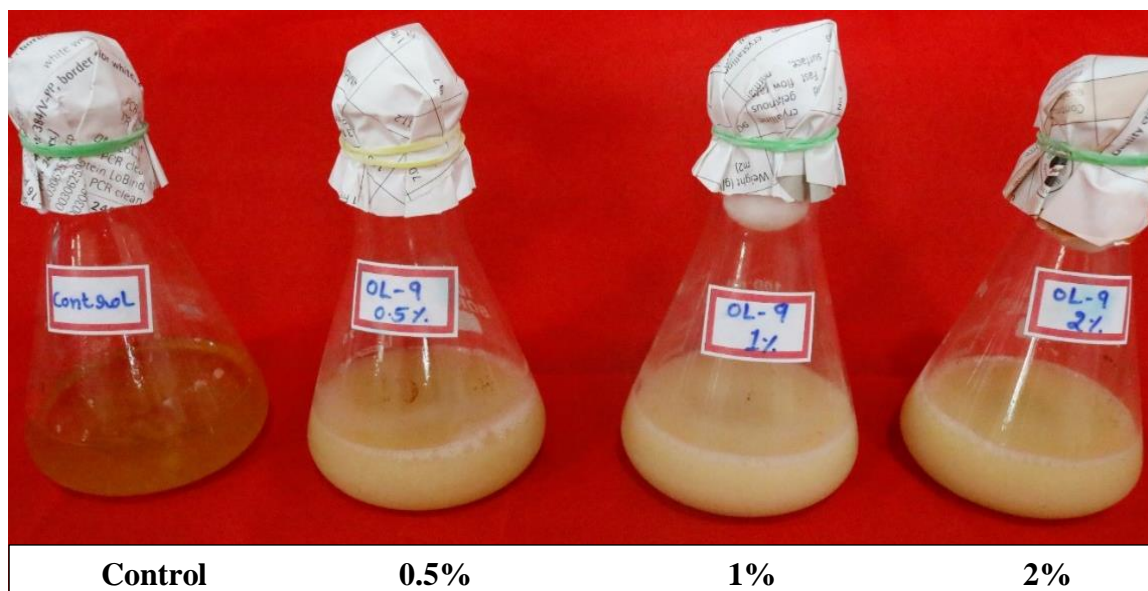


Plate 8. *In vitro* efficacy of Rose oil against *R. bataticola* in PDB

Lavender oil:

The lavender oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

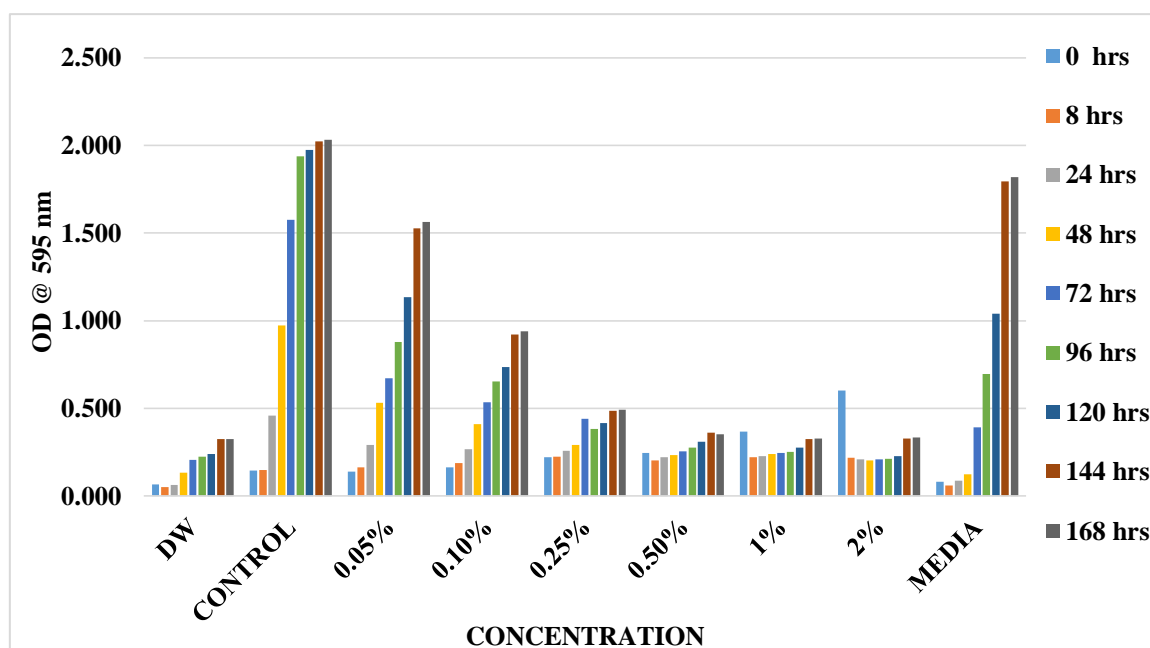


Fig 26. *In vitro* efficacy of lavender oil against *R. bataticola*

The lavender oil inhibited the *R. bataticola* growth at lower concentrations (0.05% to 0.25%) obtained was insignificant, at 0.5 per cent found was 79 per cent, at 1 per cent found was 80 per cent and at 2 per cent obtained was 83 per cent.

Clove oil:

The clove oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

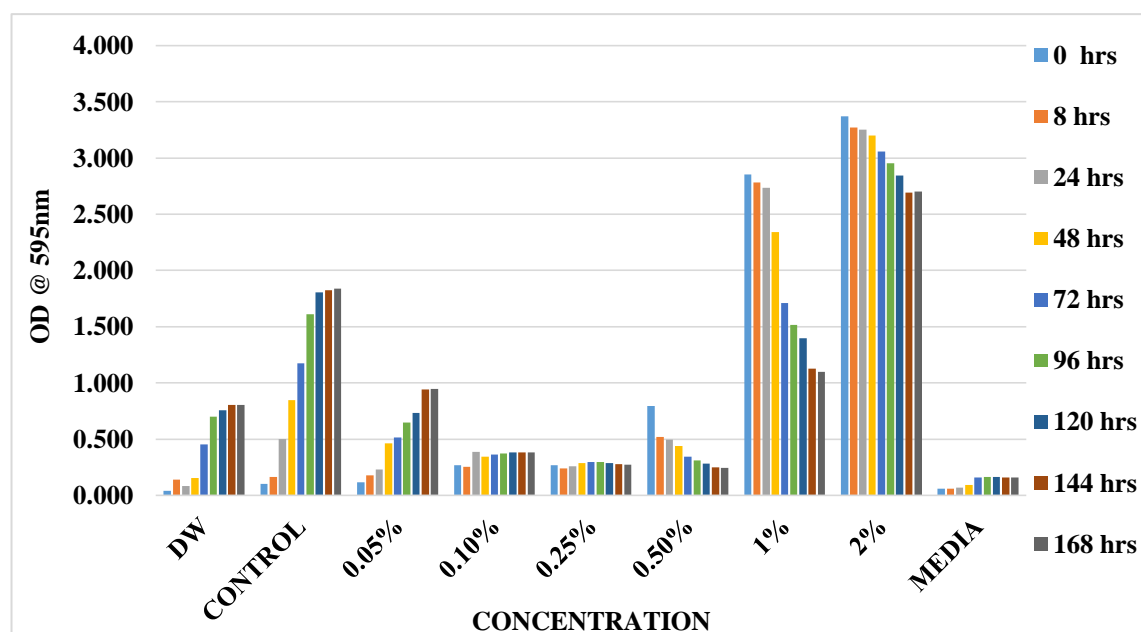


Fig 27. In vitro efficacy of clove oil against *R. bataticola*

The clove oil inhibited the *R. bataticola* growth at 0.25 and 0.5 per cent obtained was 95 and 96 per cent respectively, and at higher concentrations the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100 per cent inhibition at 1 and 2 per cent (Plate 9).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the clove oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

Cinnamon oil:

The cinnamon oil was tested for the inhibition of fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

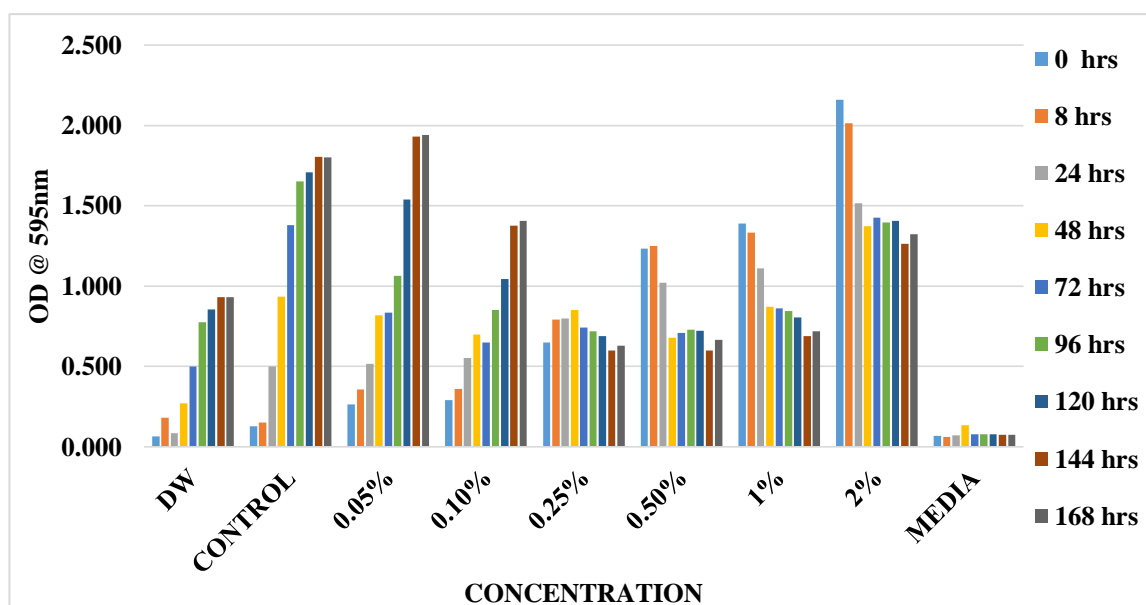


Fig 28. In vitro efficacy of cinnamon oil against *R. bataticola*

The cinnamon oil inhibited the *R. bataticola* growth at 0.25 per cent obtained was 66 per cent. As the concentration of cinnamon oil increases the wells appeared cloudy and whitish, hence it affected the spectral readings. But the visual observations confirmed that there was 100 per cent inhibition at higher concentrations (Plate 10).

The treatment evaluated was control, 0.5, 1 and 2 per cent and incubated for 7 days and observed for the growth. Complete inhibition was observed from 0.5 per cent to 2 per cent. Hence the clove oil inhibits the growth of *A. flavus* from 0.5 per cent onwards.

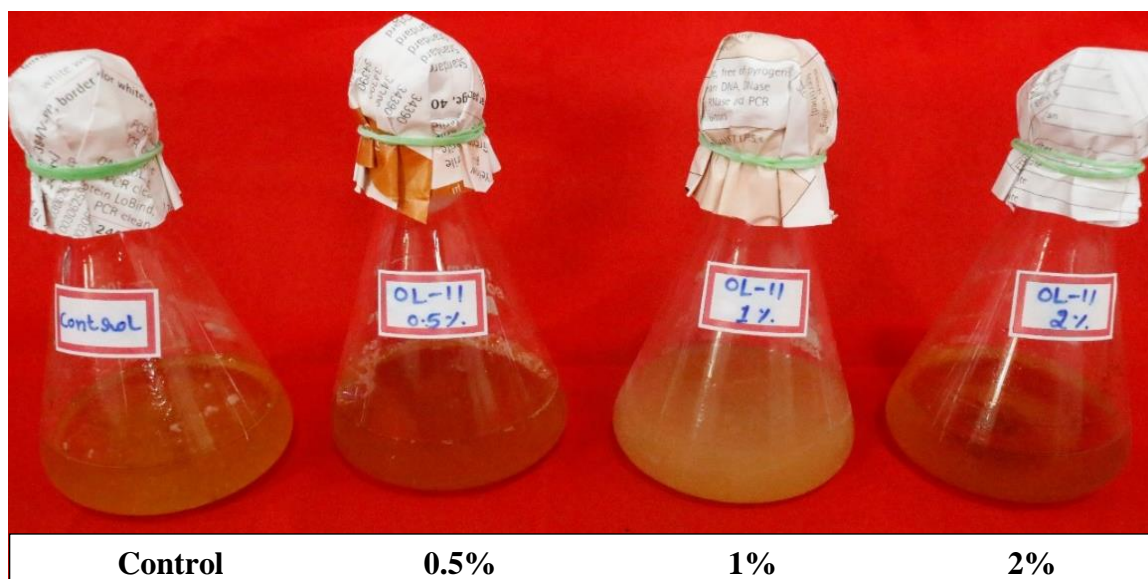


Plate 9. *In vitro* efficacy of Clove oil against *R. bataticola* in PDB

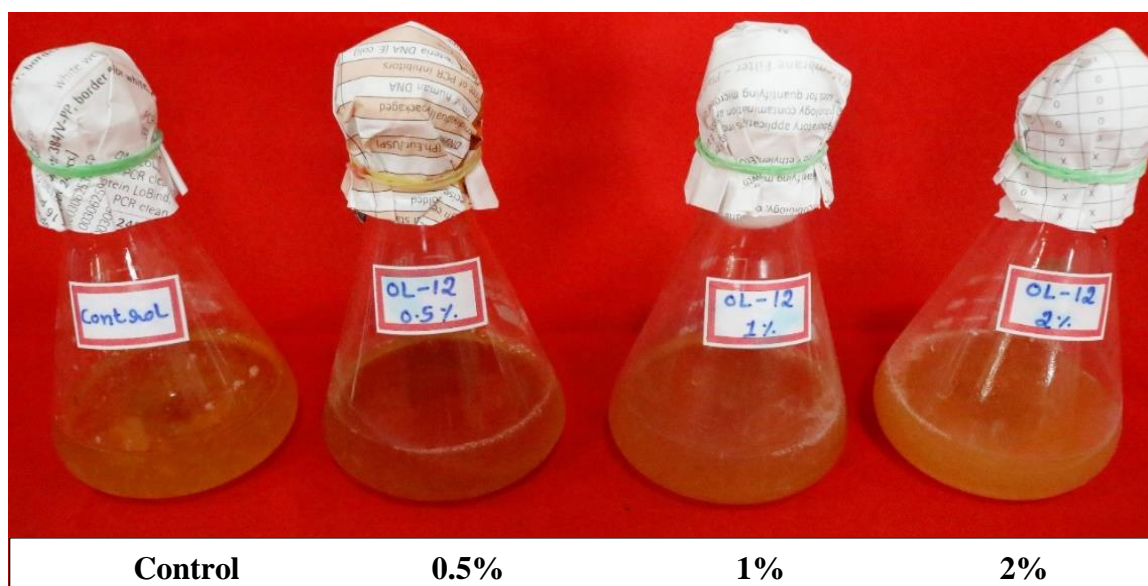


Plate 10. *In vitro* efficacy of Cinnamon oil against *R. bataticola* in PDB

Eucalyptus oil:

The eucalyptus oil was tested for the evaluation of efficacy against fungal mycelia by incubating the fungal spores at concentrations ranging from (0.05, 0.10, 0.25, 0.5, 1 and 2 per cent) for 7 days, the absorbance was measured at the OD 595 nm at different time intervals (0, 24, 48, 72, 96, 120, 144 and 168 hr) and the data recorded is presented as below.

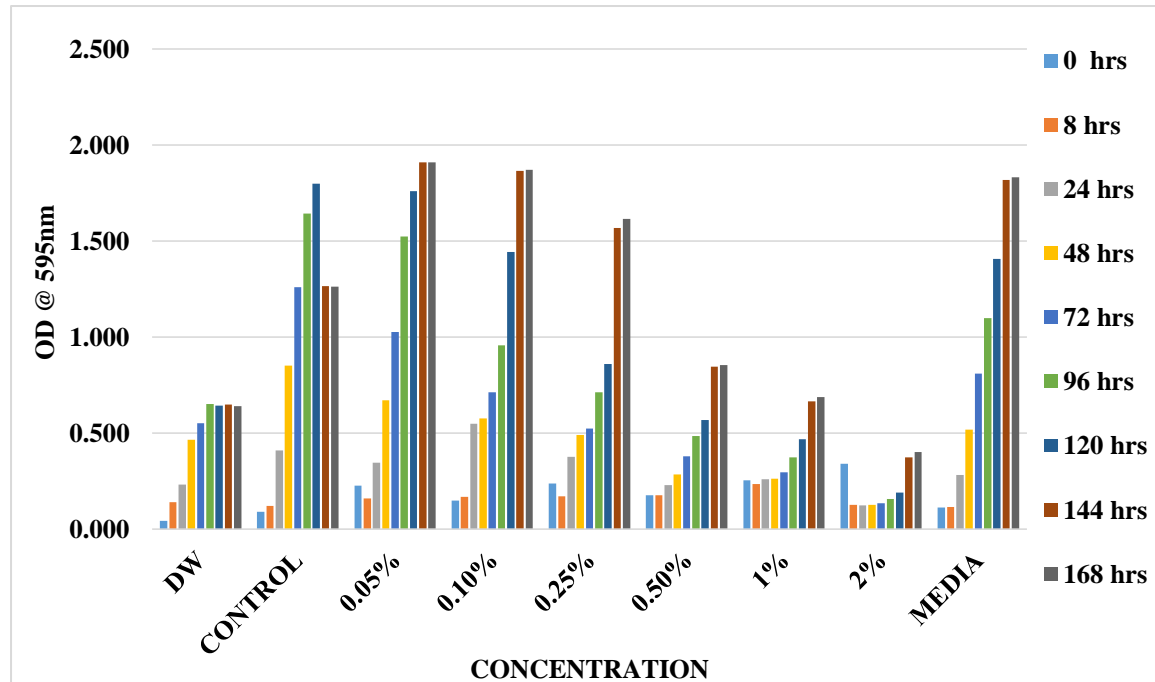


Fig 29. In vitro efficacy of eucalyptus oil against *R. bataticola*

The eucalyptus oil inhibited the *R. bataticola* growth at lower concentrations (0.05 % to 0.25 %) observed was insignificant at 0.5, 1 and 2 per cent found was 38, 45 and 68 per cent respectively.

4.1.2. Screening of essential oils against *A. flavus* and *R. bataticola* by poison food technique

4.1.2.1. *In vitro* evaluation of essential oils against *A. flavus*

Twelve essential oils were evaluated *in vitro* for their efficacy against *A. flavus* through poison food technique at 0.05, 0.1, 0.25, 0.5, 1 and 2 per cent concentrations. Among twelve essential oils tested, clove oil was found highly effective with per cent mycelia inhibition of 84.67 and 88.5 per cent at 0.05 and 0.1 per cent concentrations respectively and found 100 per cent inhibition at 0.25, 0.5, 1 and 2 per cent concentrations. Rose oil inhibited the growth of the fungus to the extent of 46.63 and 89.47 per cent at 0.05 and 0.1 per cent concentrations respectively and found 100 per cent inhibition at 0.25, 0.5, 1 and 2 per cent concentrations. Cinnamon oil inhibited the growth of the fungus to the extent of 31.4 per cent at 0.05 per cent concentration and found 100 per cent inhibition at 0.1, 0.25, 0.5, 1 and 2 per cent concentrations. Ajowain seed oil inhibited the growth of the fungus to the extent of 40.93 and 85.7 per cent at 0.05 and 0.1 per cent concentrations respectively and found 100 per cent inhibition at 0.25, 0.5, 1 and 2 per cent concentrations. Eugenol inhibited the growth of the fungus to the extent of 42.8 and 48.5 per cent at 0.05 and 0.1 per cent concentrations respectively and found 100 per cent inhibition at 0.25, 0.5, 1 and 2 per cent concentrations.

In case of orange oil insignificant inhibition was recorded at all the concentrations tested against *A. flavus*. All the concentrations of the different essential oils differed significantly in inhibiting the mycelial growth of the fungus. Among them maximum inhibition was recorded in higher concentrations (0.5, 1 and 2 %) compared to lower concentrations (0.05, 0.1 and 0.25 %).

Table 4. *In vitro* evaluation of essential oils against *A. flavus*

Sl. No.	Essential oils	Per cent inhibition*						Mean
		0.05%	0.1%	0.25%	0.5%	1%	2%	
1	Orange oil	13.27 (21.37)	5.17 (22.92)	25.67 (30.44)	30.43 (36.38)	35.17 (33.49)	36.13 (36.95)	25.97
2	Peppermint oil	30.43 (33.49)	40.00 (39.24)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	78.41
3	Citronella oil	34.20 (35.79)	47.57 (43.61)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	80.45
4	Methyl eugenol	28.50 (32.27)	48.5 (44.15)	74.2 (59.48)	100 (90.00)	100 (90.00)	100 (90.00)	75.2
5	Eugenol	42.80 (40.87)	48.5 (44.15)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	81.88
6	Ajowain seed oil	40.93 (39.78)	85.7 (67.79)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	87.77
7	Cumin seed oil	18.07 (25.16)	19.97 (26.55)	68.53 (55.88)	100 (90.00)	100 (90.00)	100 (90.00)	67.76
8	Rose oil	46.63 (43.07)	89.47 (71.07)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	89.35
9	Lavender oil	17.10 (24.43)	25.7 (30.47)	29.5 (32.90)	74.2 (59.48)	100 (90.00)	100 (90.00)	57.75
10	Clove oil	84.67 (66.95)	88.5 (70.18)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	95.53
11	Cinnamon oil	31.40 (34.09)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	88.57
12	Eucalyptus oil	15.17 (22.92)	19.97 (26.55)	22.8 (28.53)	28.5 (32.27)	34.23 (35.81)	42.8 (40.87)	27.24
		S. Em±						C. D. at 1%
Essential oil (A)		0.334						0.935
Concentration (B)		0.236						0.661
(A x B)		0.818						2.290

*Mean of three replications

Figures in the parentheses are arcsine transformed values

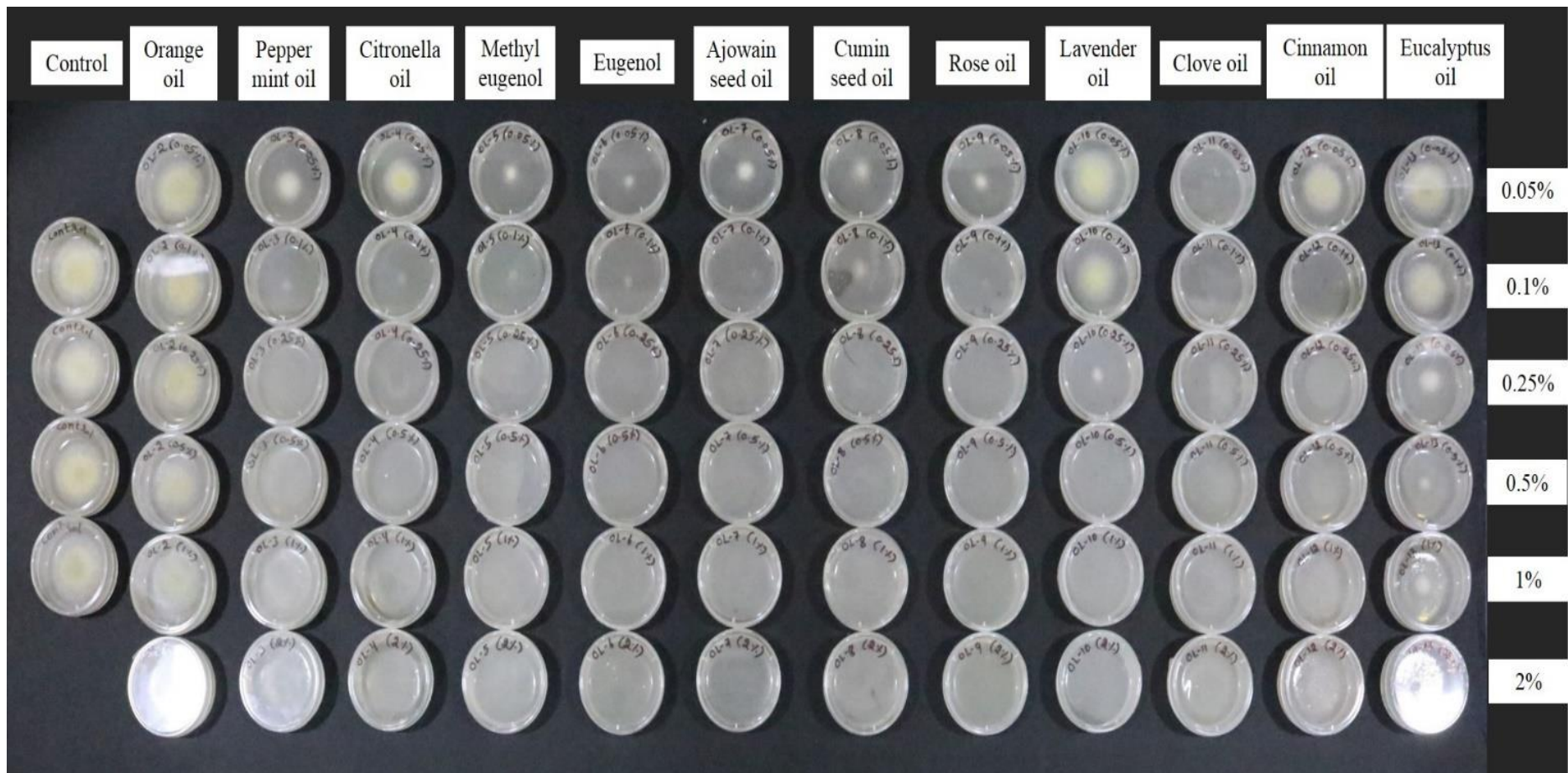


Plate 11. Inhibition of mycelial growth of *A. flavus* by essential oils under *in vitro*

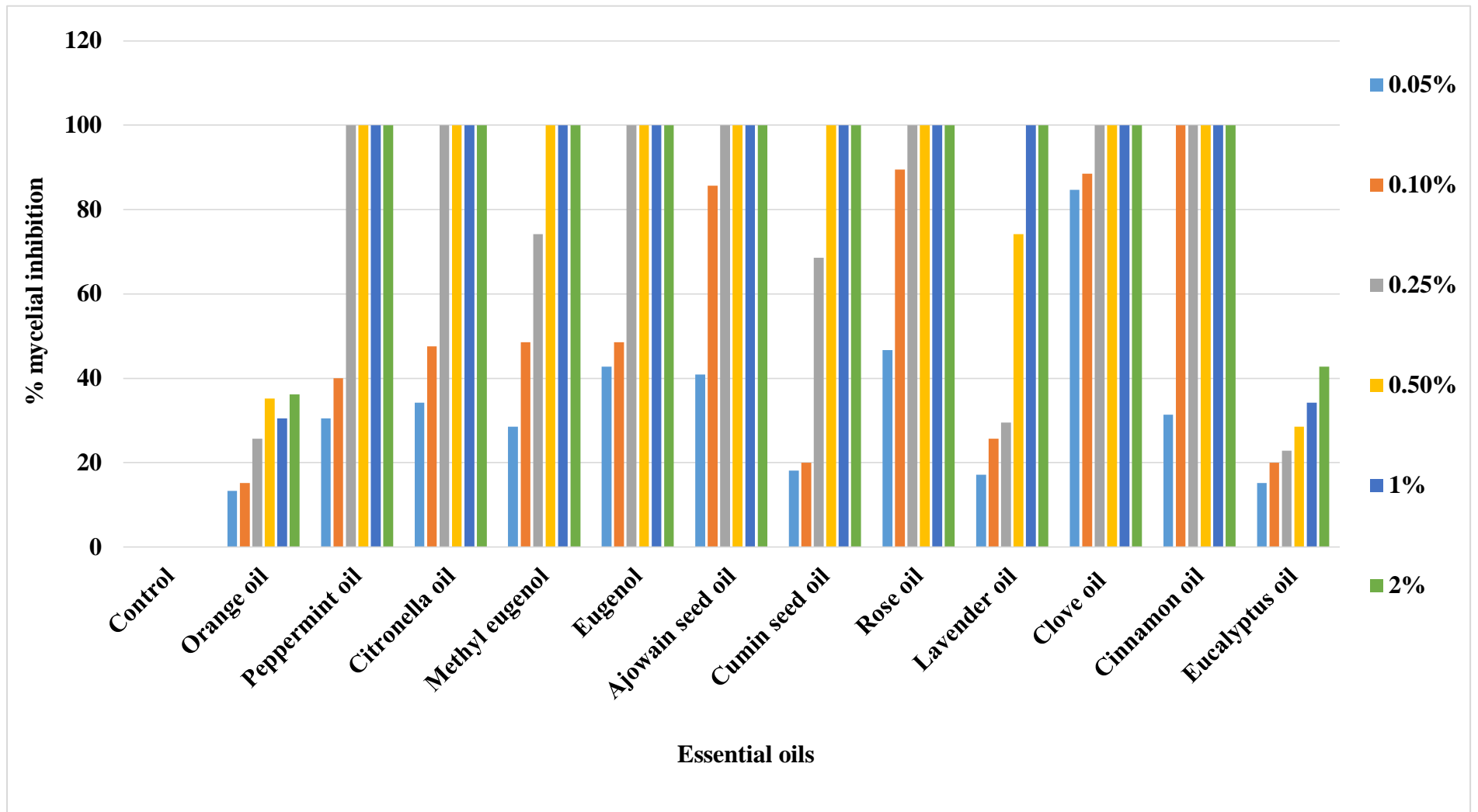


Fig 30. Inhibition of mycelial growth of *A. flavus* by essential oils under *in vitro*

4.1.2.2. *In vitro* evaluation of essential oils against *R. bataticola*

Twelve essential oils were evaluated *in vitro* for their efficacy against *R. bataticola* through poison food technique at 0.05, 0.1, 0.25, 0.5, 1 and 2 per cent concentrations. Among twelve essential oils tested, clove oil was found highly effective with per cent mycelia inhibition of 88.5 and 91.4 per cent at 0.05 and 0.1 per cent concentrations respectively and found 100 per cent inhibition at 0.25, 0.5, 1, and 2 per cent concentrations. Eugenol inhibited the growth of the fungus to the extent of 85.70 per cent at 0.05 per cent concentration and found 100 per cent inhibition at 0.1, 0.25, 0.5, 1, and 2 per cent concentrations. Ajowain seed oil inhibited the growth of the fungus to the extent of 84.73 per cent at 0.05 per cent concentration and found 100 per cent inhibition at 0.1, 0.25, 0.5, 1, and 2 per cent concentrations. Rose oil inhibited the growth of the fungus to the extent of 48.5 and 88.5 per cent at 0.05 and 0.1 per cent concentrations respectively and found 100 per cent inhibition at 0.25, 0.5, 1, and 2 per cent concentrations. Cinnamon oil inhibited the growth of the fungus to the extent of 27.57 per cent at 0.05 per cent concentration and found 100 per cent inhibition at 0.1, 0.25, 0.5, 1, and 2 per cent concentrations.

In case of orange oil insignificant inhibition was recorded at all the concentrations tested against *R. bataticola*. All the concentrations of the different essential oils differed significantly in inhibiting the mycelial growth of the fungus. Among them maximum inhibition was recorded in higher concentrations (0.5, 1 and 2 %) compared to lower concentrations (0.05, 0.1 and 0.25 %).

Table 5. *In vitro* evaluation of essential oils against *R. bataticola*

Sl. No.	Essential oil	Per cent inhibition*						Mean
		0.05%	0.1%	0.25%	0.5%	1%	2%	
1	Orange oil	8.53 (16.99)	9.47 (17.92)	10.40 (18.82)	12.33 (20.57)	24.73 (29.83)	23.77 (29.18)	14.87
2	Peppermint oil	34.20 (35.79)	42.80 (40.87)	77.10 (61.41)	100 (90.00)	100 (90.00)	100 (90.00)	75.68
3	Citronella oil	25.70 (30.47)	34.20 (35.79)	91.40 (72.95)	100 (90.00)	100 (90.00)	100 (90.00)	75.22
4	Methyl eugenol	57.10 (49.09)	60.00 (50.77)	77.10 (61.41)	91.40 (72.95)	100 (90.00)	100 (90.00)	80.93
5	Eugenol	85.70 (67.79)	100 (90)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	97.62
6	Ajowain seed oil	84.73 (67.01)	100 (90)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	97.46
7	Cumin seed oil	37.10 (37.53)	45.70 (42.54)	83.77 (66.25)	100 (90.00)	100 (90.00)	100 (90.00)	77.76
8	Rose oil	48.50 (44.15)	88.50 (70.18)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	89.5
9	Lavender oil	20.00 (26.57)	28.50 (32.27)	28.50 (32.27)	71.40 (57.68)	91.40 (72.95)	100 (90)	56.63
10	Clove oil	88.50 (70.18)	91.40 (72.95)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	99.65
11	Cinnamon oil	27.57 (31.68)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	100 (90.00)	87.93
12	Eucalyptus oil	22.80 (28.53)	31.37 (34.06)	48.50 (44.15)	48.50 (44.15)	48.50 (44.15)	48.50 (44.15)	41.36
		S. Em±						C. D. at 1%
Essential oil (A)		0.247						0.690
Concentration (B)		0.174						0.488
(A x B)		0.604						1.690

*Mean of three replications

Figures in the parentheses are arcsine transformed values

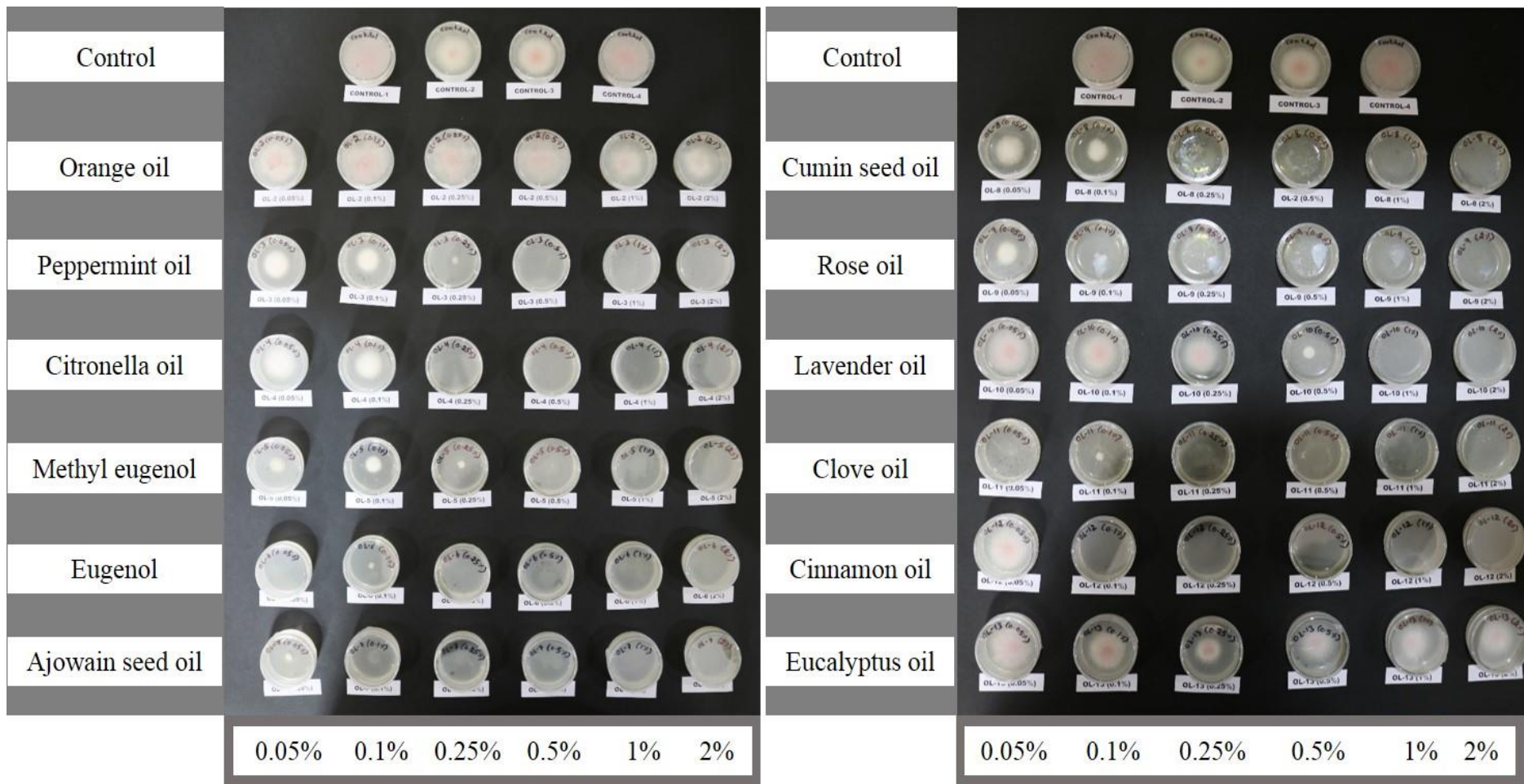


Plate 12. Inhibition of mycelial growth of *R. bataticola* by essential oils under *in vitro*

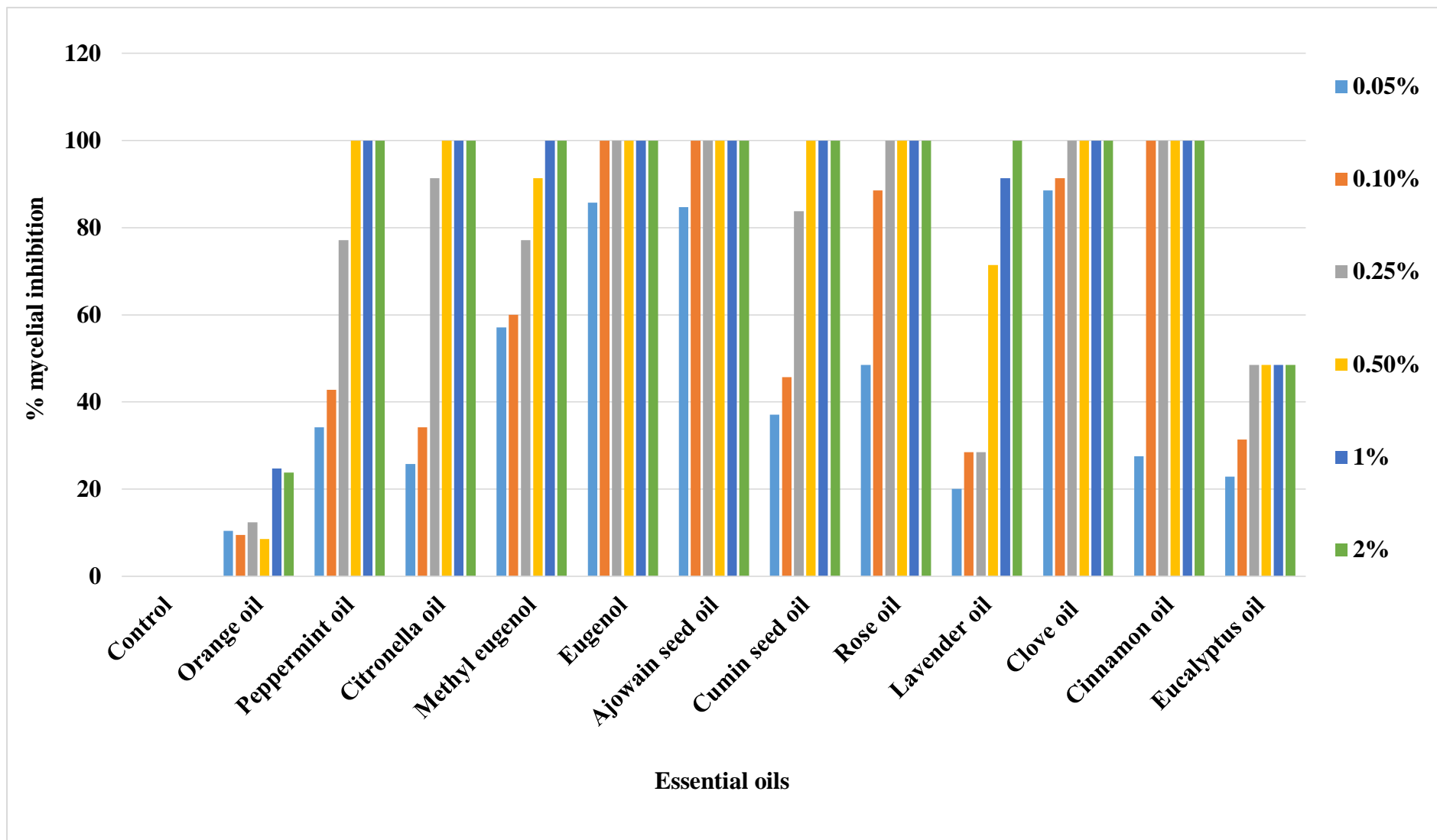


Fig 31. Inhibition of mycelial growth of *R. bataticola* by essential oils under *in vitro*

4.1.3. Resazurin assay for cell viability

4.1.3.1. Screening of essential oils against *A. flavus* and *R. bataticola* by using Resazurin assay

Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxido-reductases within viable cells. Resorufin is further reduced to hydroresorufin (uncolored and non-fluorescent). A resazurin reduction test has also been used for decades to demonstrate bacterial and yeast contamination of milk (Bigalke., 1984 and McNicholl *et al.*, 2006).

The effectiveness of this modified resazurin assay has been demonstrated with essential oils, plant extracts and chemical entities and the results were furnished below (Plate 13).

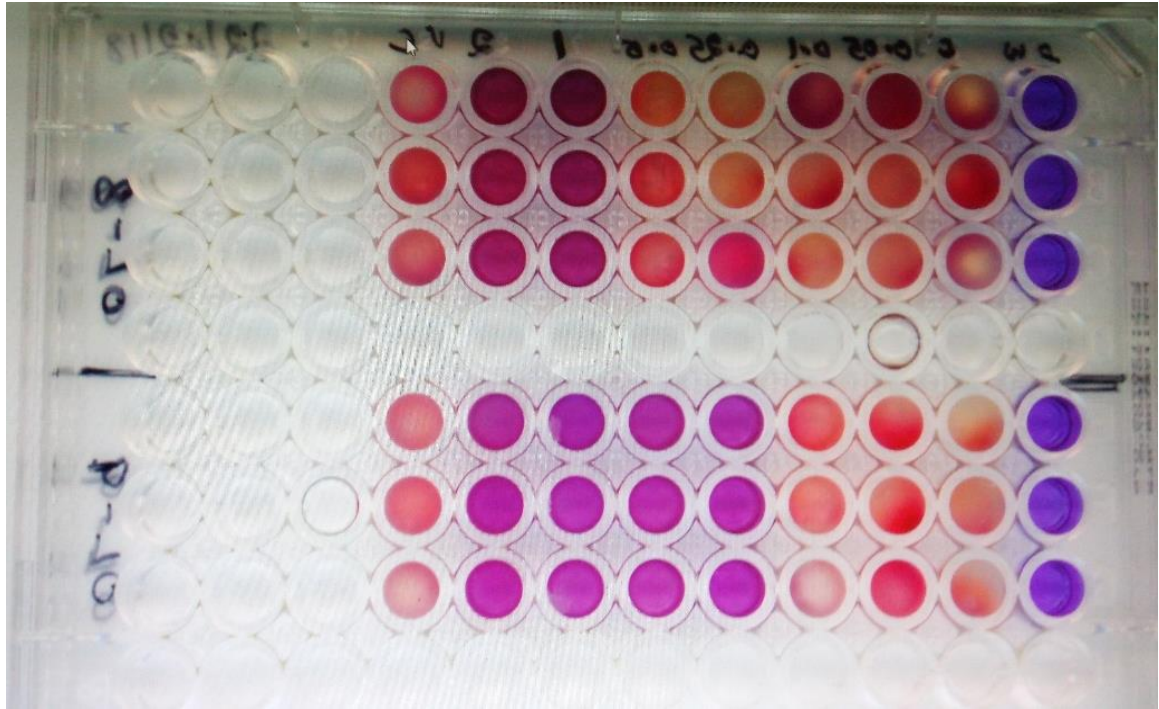


Plate 13. Resazurin Assay

Purple - Dead

Pink/Colorless - Live

4.1.4. Pyocyanin assay

4.1.4.1. Pyocyanin assay in *A. flavus*

Pyocyanin assay was carried out by incubating the essential oils at doses ranging from (0.5, 1 and 2 %) for 5 days and assay was carried out on 5th day after the exposure to know the inhibiting ability of quorum sensing.

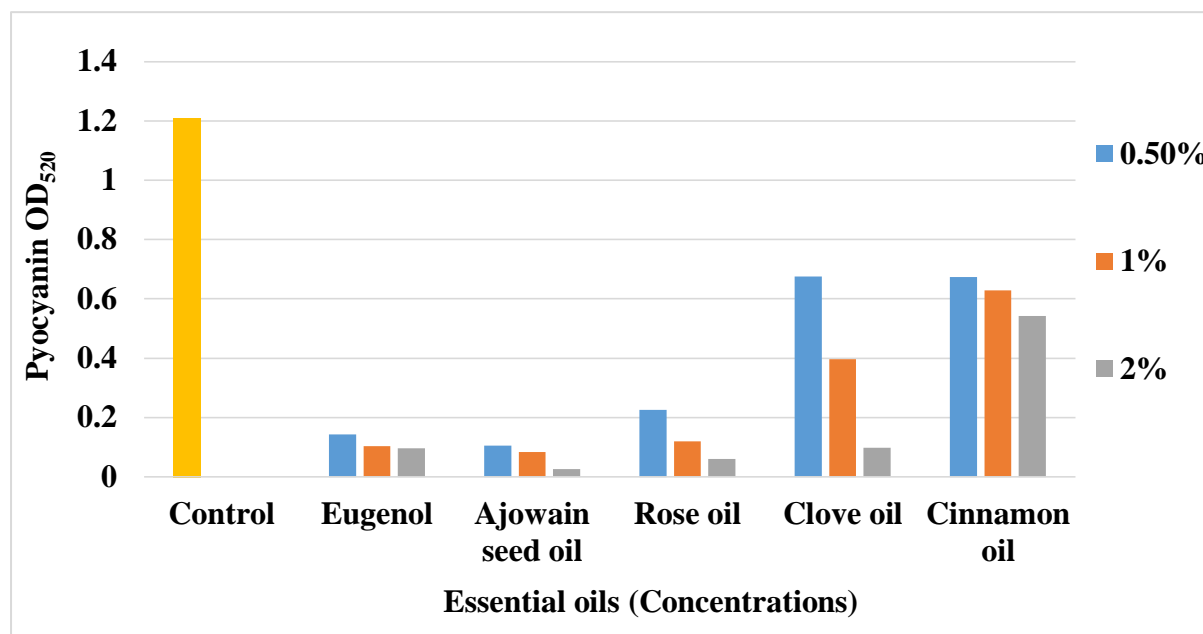


Fig 32. Inhibition of pyocyanin production in *A. flavus* by Essential oils.

Essential oils showed reduction in pyocyanin production in *A. flavus*. The Eugenol, Ajowain seed oil and Rose oil showed significant reduction of Pyocyanin at different concentrations. In Eugenol treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 88, 91 and 92 per cent respectively. In Ajowain seed oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 91, 93 and 98 per cent respectively. In Rose oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 81, 90 and 95 per cent respectively. In Clove oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 44, 67 and 92 per cent respectively. In Cinnamon oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 44, 48 and 55 per cent respectively.

4.1.4.2. Pyocyanin assay in *R. bataticola*

Pyocyanin assay was carried out by incubating the essential oils at doses ranging from (0.5, 1 and 2 %) for 5 days and assay was carried out on 5th day after the exposure to know the inhibiting ability of quorum sensing.

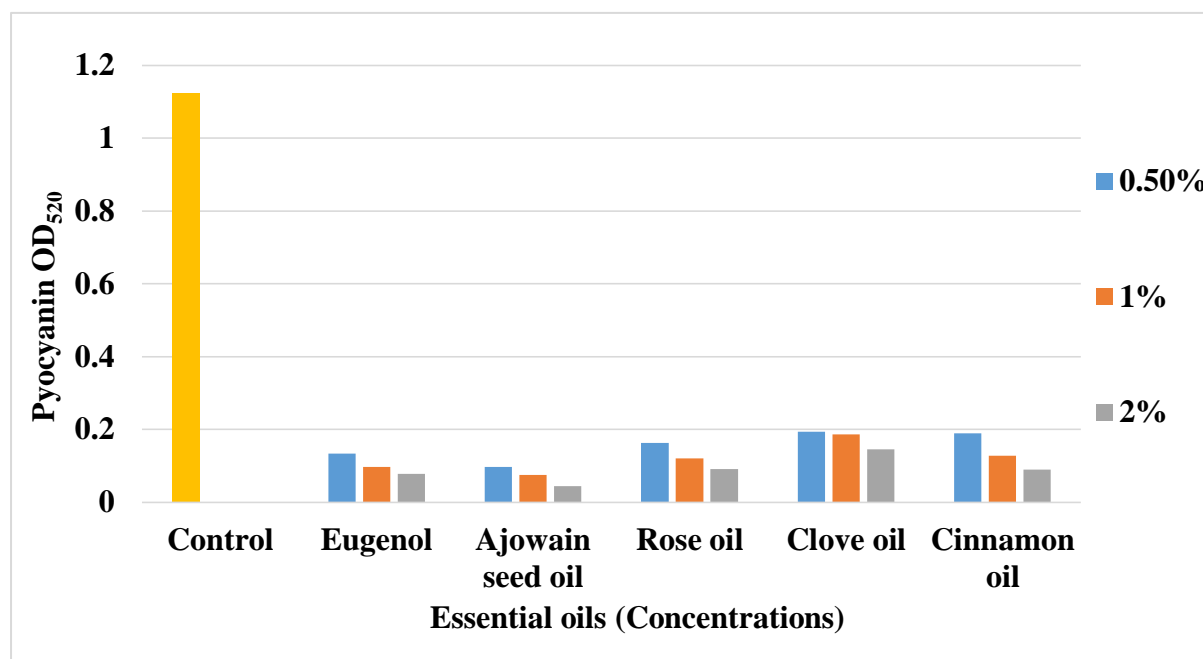


Fig 33. Inhibition of pyocyanin production in *R. bataticola* by Essential oils

Essential oils showed reduction in pyocyanin production in *R. bataticola*. In Eugenol treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 88, 91 and 93 per cent respectively. In Ajowain seed oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 91, 93 and 96 per cent respectively. In Rose oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 85, 89 and 92 per cent respectively. In Clove oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 83, 84 and 87 per cent respectively. In Cinnamon oil treated, at 0.5, 1 and 2 per cent the reduction of Pyocyanin production found was 83, 88 and 92 per cent respectively.

4.1.5. Estimation of peroxidase (POX) activity

4.1.5.1. Estimation of peroxidase (POX) activity in *A. flavus*

During the course of investigation, a decrease in peroxidase activity was observed during the course of time. Decrease in peroxidase activity is attributed to the loss of peroxidase enzyme during stress condition. The peroxidase activity in control was decreased from 4.282 U/ml/min to 2.191 U/ml/min when the time period was increased from 4 hr to 24 hr. Similarly in eugenol treated it was found that the decrease from 3.1 U/ml/min to 1.644 U/ml/min, in ajowain seed oil treated it was found that the decrease from 7.093 U/ml/min to 3.368 U/ml/min, in rose oil treated it was found that the decrease from 6.544 U/ml/min to 4.018 U/ml/min, in clove oil treated it was found that the decrease from 3.128 U/ml/min to 1.762 U/ml/min and in cinnamon oil treated it was found that the decrease from 6.795 U/ml/min to 3.324 U/ml/min. The data presented (Fig 34) reveal the effects of the different essential oils exposure on the peroxidase activity in the *A. flavus* culture.

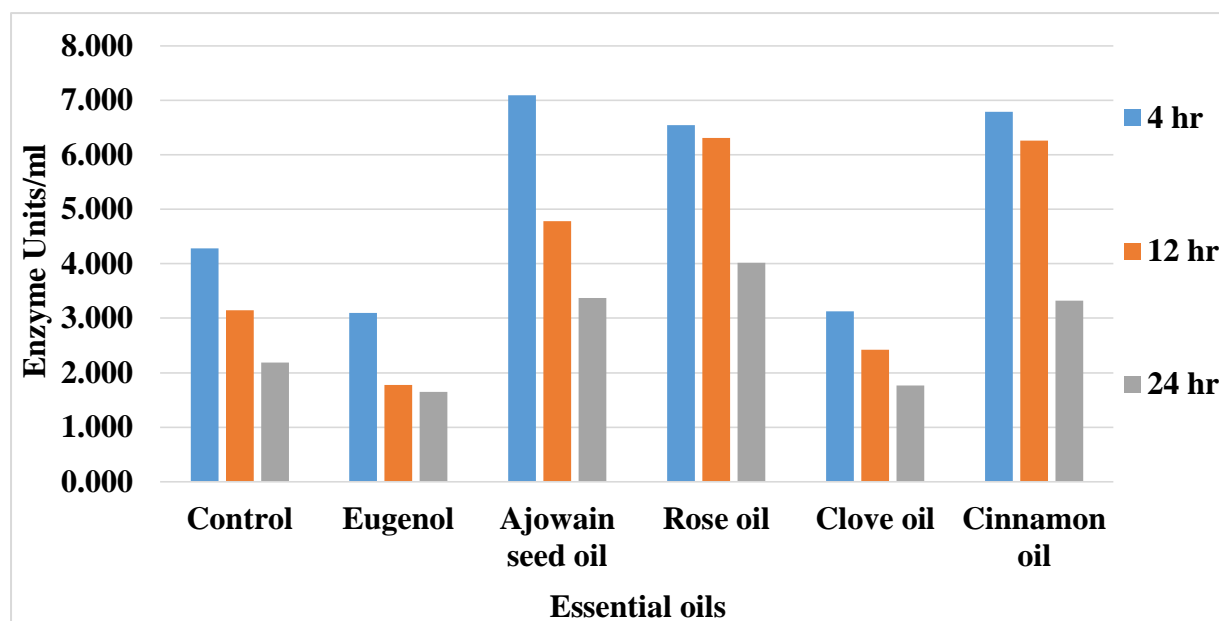


Fig 34. Effect of different essential oils on peroxidase activity in *A. flavus* culture

4.1.5.2. Estimation of peroxidase (POX) activity in *R. bataticola*

During the course of investigation, a decrease in peroxidase activity was observed during the course of time. Decrease in peroxidase activity is attributed to the loss of peroxidase enzyme during stress condition. The peroxidase activity in control was decreased from 16.931 U/ml/min to 7.084 U/ml/min when the time period was increased from 4 hr to 24 hr. Similarly in eugenol treated it was found that the decrease from 3.01 U/ml/min to 1.896 U/ml/min, in ajowain seed oil treated it was found that the decrease from 7.758 U/ml/min to 3.527 U/ml/min, in rose oil treated it was found that the decrease from 4.64 U/ml/min to 3.759 U/ml/min, in clove oil treated it was found that the decrease from 4.683 U/ml/min to 3.049 U/ml/min and in cinnamon oil treated it was found that the decrease from 3.715 U/ml/min to 1.701 U/ml/min. The data presented (Fig.35) reveal the effects of the different essential oils exposure on the peroxidase activity in the *R. bataticola* culture.

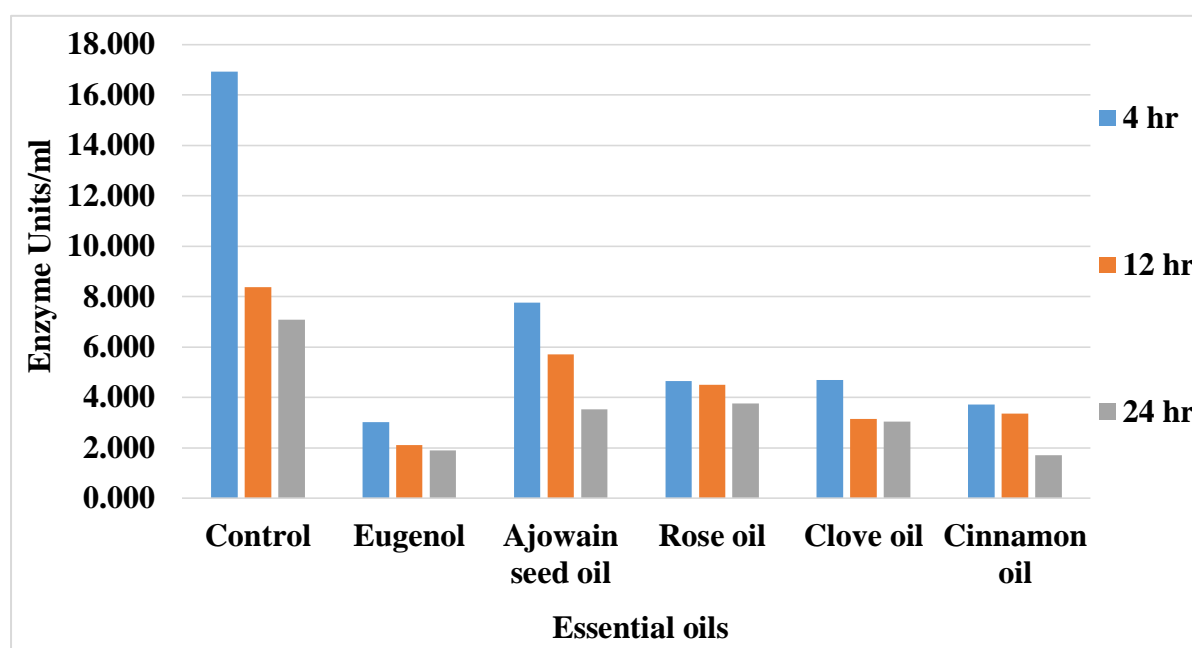


Fig 35. Effect of different essential oils on peroxidase activity in *R. bataticola* culture

4.1.6. Estimation of catalase (CAT) activity

4.1.6.1. Estimation of catalase (CAT) activity in *A. flavus*

During the course of investigation, an increase in catalase activity was observed during the course of time. Increase in catalase activity is attributed to the accumulation of catalase enzyme during stress condition. The catalase activity in control was increased from 0.247 U/ml/min to 0.626 U/ml/min when the time period was increased from 4 hr to 24 hr. Similarly in eugenol treated it was found that the decrease from 0.142 U/ml/min to 0.482 U/ml/min, in ajowain seed oil treated it was found that the decrease from 0.362 U/ml/min to 0.671 U/ml/min, in rose oil treated it was found that the decrease from 0.281 U/ml/min to 0.595 U/ml/min, in clove oil treated it was found that the decrease from 0.062 U/ml/min to 0.151 U/ml/min and in cinnamon oil treated it was found that the decrease from 0.182 U/ml/min to 0.280 U/ml/min. The data presented (Fig.36) reveal the effects of the different essential oils exposure on the catalase activity in the *R. bataticola* culture.

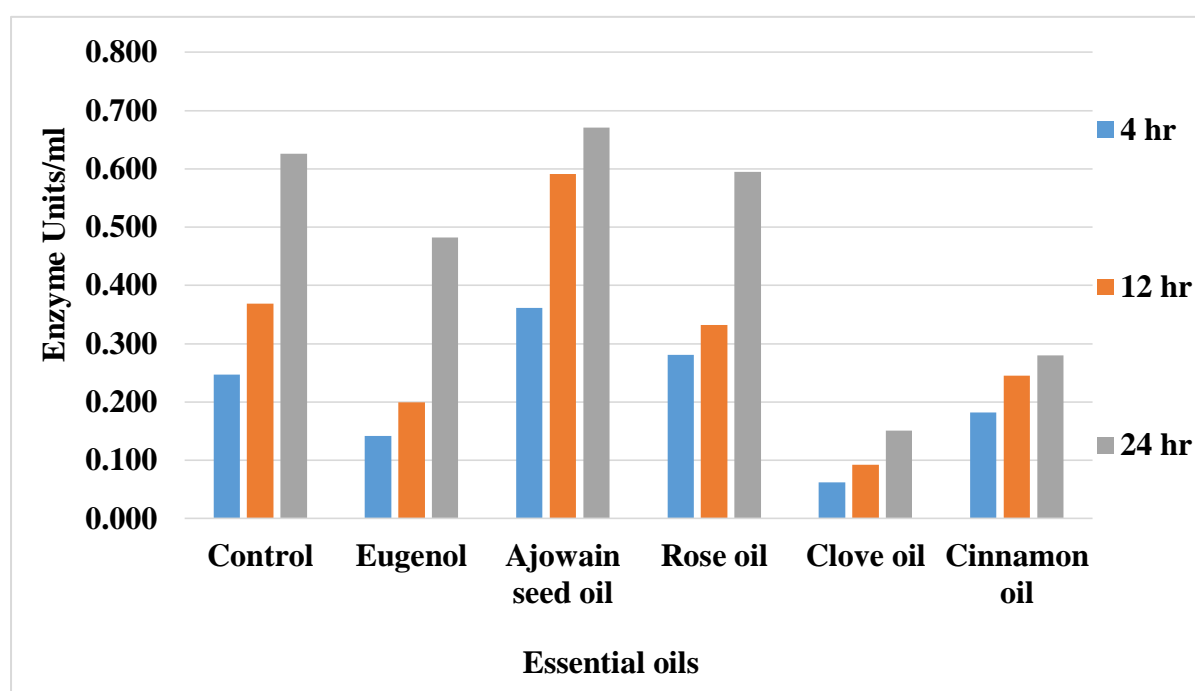


Fig 36. Effect of different essential oils on catalase activity in *A. flavus* culture

4.1.6.2. Estimation of catalase (CAT) activity in *R. bataticola*

During the course of investigation, an increase in catalase activity was observed during the course of time. Increase in catalase activity is attributed to the accumulation of catalase enzyme during stress condition. The catalase activity in control was increased from 0.247 U/ml/min to 0.626 U/ml/min when the time period was increased from 4 hr to 24 hr. Similarly in eugenol treated it was found that the decrease from 0.142 U/ml/min to 0.482 U/ml/min, in ajowain seed oil treated it was found that the decrease from 0.362 U/ml/min to 0.671 U/ml/min, in rose oil treated it was found that the decrease from 0.281 U/ml/min to 0.595 U/ml/min, in clove oil treated it was found that the decrease from 0.062 U/ml/min to 0.151 U/ml/min and in cinnamon oil treated it was found that the decrease from 0.182 U/ml/min to 0.280 U/ml/min. The data presented (Fig.37) reveal the effects of the different essential oils exposure on the catalase activity in the *R. bataticola* culture.

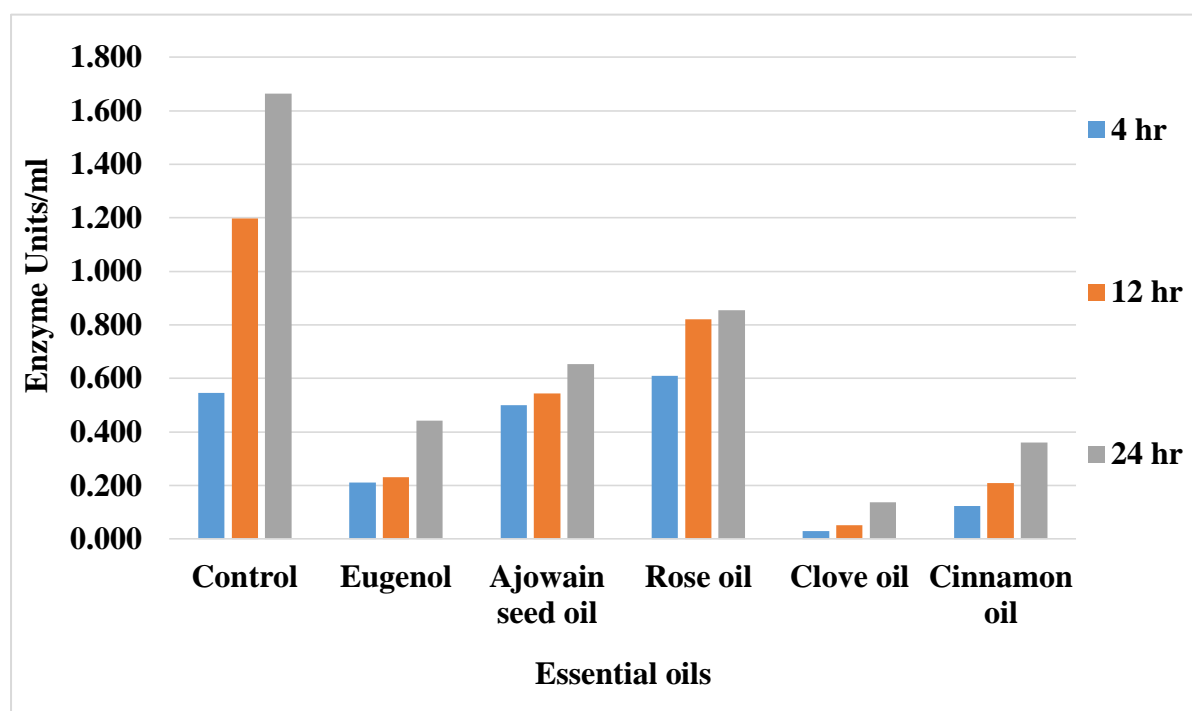


Fig 37. Effect of different essential oils on catalase activity in *R. bataticola* culture

Table 6. Essential oils tested against *A. flavus* and their results

Sl. No.	Essential oils	Concentrations					
		0.05%	0.1%	0.25%	0.5%	1%	2%
1	Orange oil	-	-	-	-	-	-
2	Peppermint oil	-	-	+	+	+	+
3	Citronella oil	-	-	+	+	+	+
4	Methyl eugenol	-	-	-	+	+	+
5	Eugenol	+	+	+	+	+	+
6	Ajowain seed oil	+	+	+	+	+	+
7	Cumin seed oil	-	-	-	+	+	+
8	Rose oil	+	+	+	+	+	+
9	Lavender oil	-	-	-	-	+	+
10	Clove oil	+	+	+	+	+	+
11	Cinnamon oil	+	+	+	+	+	+
12	Eucalyptus oil	-	-	-	-	-	-

+ 100% inhibition

- No inhibition

Table 7. Essential oils tested against *R. bataticola* and their results

Sl. No.	Essential oils	Concentrations					
		0.05%	0.1%	0.25%	0.5%	1%	2%
1	Orange oil	-	-	-	-	-	-
2	Peppermint oil	-	-	-	+	+	+
3	Citronella oil	-	-	-	+	+	+
4	Methyl eugenol	-	-	-	-	+	+
5	Eugenol	+	+	+	+	+	+
6	Ajowain seed oil	+	+	+	+	+	+
7	Cumin seed oil	-	-	-	+	+	+
8	Rose oil	+	+	+	+	+	+
9	Lavender oil	-	-	-	-	-	+
10	Clove oil	+	+	+	+	+	+
11	Cinnamon oil	+	+	+	+	+	+
12	Eucalyptus oil	-	-	-	-	-	-

+ 100% inhibition

- No inhibition

The synthetic chemicals tested has not shown the inhibition of mycelial growth in both *A. flavus* and *R. bataticola* fungus on testing with 96-well micro titer plate and Resazurin assay. The data is presented in tabular form (Table 8, 9, 10, 11 and 12).

Table 8. Activity of Lactone derived synthetic chemicals tested (I-Batch)

Sl. No.	Synthetic chemicals (I-Batch)	Concentrations		
		1 ppm	10 ppm	100 ppm
1	4a	-	-	-
2	4b	-	-	-
3	4c	-	-	-
4	5a	-	-	-
5	5b	-	-	-
6	5c	-	-	-
7	6a	-	-	-
8	6b	-	-	-
9	6c	-	-	-
10	7a	-	-	-
11	7b	-	-	-
12	7c	-	-	-
13	8a	-	-	-
14	8b	-	-	-
15	8c	-	-	-

-Inhibition was not observed

Table 9. Activity of Lactone derived synthetic chemicals tested (II-Batch)

Sl. No.	Synthetic chemicals (II-Batch)	Concentrations		
		1 ppm	10 ppm	100 ppm
1	5a	-	-	-
2	5b	-	-	-
3	5c	-	-	-
4	10a	-	-	-
5	10b	-	-	-
6	10c	-	-	-
7	10d	-	-	-
8	10e	-	-	-
9	10f	-	-	-

-Inhibition was not observed

Table 10. Activity of Lactone derived synthetic chemicals tested (III-Batch)

Sl. No.	Synthetic chemicals (III-Batch)	Concentrations		
		1 ppm	10 ppm	100 ppm
1	6a	-	-	-
2	6b	-	-	-
3	6c	-	-	-
4	7a	-	-	-
5	7b	-	-	-
6	7c	-	-	-
7	8a	-	-	-
8	8b	-	-	-
9	8c	-	-	-
10	9a	-	-	-
11	9b	-	-	-
12	10a	-	-	-
13	10b	-	-	-
14	11a	-	-	-
15	11b	-	-	-

-Inhibition was not observed

Table 11. Activity of Lactone derived synthetic chemicals tested (IV-Batch)

Sl. No.	Synthetic chemicals (IV-Batch)	Concentrations		
		1 ppm	10 ppm	100 ppm
1	7a	-	-	-
2	7b	-	-	-
3	7c	-	-	-
4	7d	-	-	-
5	7e	-	-	-
6	7f	-	-	-
7	7g	-	-	-
8	7h	-	-	-
9	7i	-	-	-
10	7j	-	-	-
11	7k	-	-	-
12	7l	-	-	-
13	7m	-	-	-
14	7n	-	-	-
15	7o	-	-	-

-Inhibition was not observed

Table 12. Activity of Lactone derived synthetic chemicals tested (V-Batch)

Sl. No.	Synthetic chemicals (V-Batch)	Concentrations		
		1 ppm	10 ppm	100 ppm
1	7a	-	-	-
2	7b	-	-	-
3	7c	-	-	-
4	7d	-	-	-
5	7e	-	-	-
6	8a	-	-	-
7	8b	-	-	-
8	8c	-	-	-
9	8d	-	-	-
10	8e	-	-	-

-Inhibition was not observed

Table 13. Plant extracts tested and their results

Sl. No.	Plant Extracts	Code	Concentrations		
			0.5%	1%	2%
1	<i>Azadirachta indica</i> leaf	1	-	-	-
2	<i>Centella asiatica</i>	2	-	-	-
3	<i>Ocimum gratissimum</i>	3	-	-	-
4	<i>Ocimum tenuiflorum</i>	4	-	-	-
5	<i>Aeonium arboreum</i>	5	-	-	-
6	<i>Styrax spp.</i>	6	-	-	-
7	<i>Laguncularia recemosa</i>	7	-	-	-
8	<i>Euphrasia officinalis</i>	8	-	-	-
9	<i>Tecoma stans</i>	9	-	-	-
10	<i>Duranta erecta</i>	10	-	-	-
11	<i>Urtica dioica</i>	11	-	-	-
12	<i>Morinda citrifolia</i>	12	-	-	-
13	<i>Justicia adhatoda</i>	13	-	-	-
14	<i>Vitex nigundo</i>	14	-	-	-
15	<i>Vinca major</i>	15	-	-	-
16	<i>Lawsonia inermis</i>	16	-	-	-
17	<i>Gloriosa superba</i>	17	-	-	-
18	<i>Lonicera japonica</i>	18	-	-	-
19	<i>Aloe vera</i>	20	-	-	-
20	<i>Baccharis trimera</i>	21	-	-	-
21	<i>Cordia verbenacea</i>	22	-	-	-
22	<i>Euphorbia tirucalli</i>	23	-	-	-
23	<i>Juglans regia</i>	24	-	-	-
24	<i>Leonotis nepetaefolia</i>	25	-	-	-

-Inhibition was not observed

The plant extracts obtained from Jain University in coded form was tested for mycelial growth in both *Aspergillus flavus* and *R. bataticola* fungus on testing with 96 well microtiter plate with PDB medium and Resazurin assay. Inhibition of growth was not observed in all the tested samples. The data is presented in tabular form (Table 13).

V. DISCUSSION

After the advent of first chemical to control plant diseases, use of chemicals in managing plant diseases was being exploited because of its rapid action and quick results. Instead of their excellent effect on plant pathogens they may leave residual toxic effect if used in excess of recommended quantity. Moreover, they also result in development of chemical resistant strains of the pathogens. Most of researchers till date gave main emphasis on controlling plant pathogens by using chemicals, only few researchers used other alternative strategies for controlling plant pathogens including plant product preparations. Recently, the scientific interest in biological properties of essential oils has been increased. New researches about biological active secondary compounds present in essential oils of plants have been seen as a potential way to control fungal contamination (Burt, 2004; Soliman and Badea, 2002; Tajkarimi *et al.*, 2010).

The environmental consciousness on the part of scientists and general public has drawn attention to the increasing amount of fungicides and pesticides used on crop plant and their harmful effect on flora and fauna. These resulted in search for bio-degradable products which can be effective in the management of diseases without any toxic effect. Chemical hazards and development of fungicide resistant strain of pathogens call for advent of alternative strategies and natural plant preparations can replace the synthetic fungicides effectively.

A. flavus is an opportunistic pathogen of crops. It is important because it produces aflatoxin as a secondary metabolite in the seeds of a number of crops both before and after harvest (Maren., 2007). Aflatoxin is a potent carcinogen that is highly regulated in most countries. In the field, aflatoxin is associated with drought-stressed oilseed crops including maize, peanut, cotton seed and tree nuts. Under the right conditions, the fungus will grow and produce aflatoxin in almost any stored crop seed. In storage, aflatoxin can be controlled by maintaining available moisture at levels below that which will support growth of *A. flavus*. A number of field control measures are being utilized or explored, including: modification of cultural practices, development of resistant crops through molecular and proteomic techniques, competitive exclusion using strains that do not produce aflatoxin and development of field treatments that would block aflatoxin production. In the present study we found the effective essential oils which are able to control the *A. flavus* growth *in vitro*.

The present investigation was undertaken to evaluate essential oils, plant extracts and synthetic chemicals. Among these 12 essential oils showed the inhibition ranging from 10 -100 % and concentrations tested were from 0.05% to 2 %, minimum inhibition of mycelial growth was recorded 9% with orange oil at 2% incubation and maximum inhibition of 100% growth was recorded at 2 % in all the test essential oils except Orange oil and eucalyptus oil. Among these essential oils eugenol, ajowain seed oil, rose oil, clove oil and cinnamon oil were effective in controlling *A. flavus* and *R. bataticola* in micro titer plate method, poison food technique and resazurin assay. These all the three methods confirmed the control of fungal spores.

There was a significant difference between eugenol, ajowain seed oil, rose oil, clove oil and cinnamon oil than the other essential oils against *A. flavus* and *R. bataticola*. And also the other essential oils also inhibited the growth of *A. flavus* and *R. bataticola* to various degrees. Similarly, Fernanda *et al.*, (2012) observed that ginger oil inhibited the mycelial growth of *A. flavus*. Also reported that fennel and thyme essential oils were most toxic against *A. flavus*.

Evaluation of the antifungal properties of the essential oils *in vitro*

Based on the information provided in the literature, twelve essential oils were selected for these experiments to determine their potential antifungal activity against *A. flavus* and *R. bataticola* pathogens.

Among twelve essential oils five essential oils completely suppressed pathogen growth in both pathogens within the 168 hr period of the experiments starting from 24 hr. These oils were eugenol, ajowain seed oil, rose oil, clove oil and cinnamon oil. Although other essential oils could significantly suppress growth of the pathogen, only five could completely suppress growth of both pathogens. Twelve oils were able to significantly suppress *A. flavus* and *R. bataticola* mycelium growth.

In poison food technique eugenol, ajowain seed oil, rose oil, clove oil and cinnamon oil were checked for the growth of *A. flavus* and *R. bataticola*, at 0.5, 1 and 2 per cent concentrations and recorded the 100 per cent inhibition after 7 days of incubation.

The twelve essential oils chosen for these experiments also had varying degrees of antifungal activity. Burt (2004) also reported that the essential oils have inherent amount

of activity and their composition depends on the methods of extraction and distillation and the part of the plant from where the oils were extracted. Essential oils of consistent composition and antifungal activity are difficult to obtain because oil composition changes from plant to plant and year to year (Burt, 2004). This variation can be problematic when using the essential oils as seed treatments. However, some of the variation can be managed if the precise composition of the essential oils is known. GC-MS Gas Chromatography Mass Spectrometry analysis can be used as a tool to estimate and possibly remediate these differences in essential oils composition. Once the constituent compounds of the oils are known, their antifungal activity can be compared to other results obtained from oils of slightly different composition.

The essential oils capable of controlling both pathogens during the entire length of this experiment were selected for assessing the minimum inhibitory concentration of the essential oils. These five oils were eugenol, ajowain seed oil, rose oil, clove oil and cinnamon oil.

Thyme essential oil showed better antifungal effect against *A. flavus* and *A. parasiticus*. This antifungal activity is reported in several studies. Soliman and Badea (2002) reported that thyme essential oil (≤ 500 ppm) completely inhibited the growth of *A. flavus* and *A. parasiticus*. This same effect was observed by Nguefack *et al.* (2004), that the thyme oil at a concentration of 200 ppm reduced the radial growth of *A. flavus* by 81 %. Complete inhibition of conidial germination of *A. flavus* was achieved by 1000 ppm. Among various plants tested, thyme essential oil inhibited growth and aflatoxin production of *A. parasiticus* (Razzaghi-Abyaneh *et al.*, 2009). The same effect was observed for *A. flavus* (Kumar *et al.*, 2008).

In vitro screening of some essential oils against *Fusarium oxysporum* Schlecht and *F. udum* Butler which cause wilt diseases of pigeon pea (*Cajanus cajan* (L.) Millsp.) Revealed that the oil of *Trachyspermum ammi* (ajowain) was a strong fungi toxicant. The toxicity of the oil was found to be fungicidal at 0.1 %, which inhibited heavy doses of inoculum (25 fungal discs, each of 5-mm diameter) and killed the test pathogen in just 2-3 days. The oil did not have any phytotoxic effects on seed germination and seedling growth of the host plant, *C. cajan*. The essential oil's toxicity prevents the wilt disease of pigeon pea at 10 % concentration. It has also been reported to be harmless to human health.

Apple was affected by gray mould caused by *Botrytis italics* and blue mould caused by *Penicillium spp.* was the most important diseases attacking apple fruit during storage Latif and Fatein. (2016). Evaluated the effects of some essential oils *i.e.*, eucalyptus, lemon grass and thyme on linear growth and spore. The highest reduction was obtained with eucalyptus and thyme at concentrations of 0.6 per cent and lemon grass at 0.8 per cent which reduced both diseases incidence and rotted part tissue more than 74 per cent and 75.8 per cent, respectively. Meanwhile, other treatments showed moderate effect. It could be suggested that essential oils could make them an excellent treatment for controlling postharvest diseases of apple fruits. But eucalyptus oil was not found to be effective against *A. flavus* and *R. bataticola* *in vitro*.

Antifungal activity of different concentrations of the essential oil was evaluated using disc diffusion method. The most abundant compounds identified in the essential oil were 1, 8-cineole (16.2 %), α -pinene (15.6 %), α -phellandrene (10.0 %), and p-cymene (8.1 %). The EO produced complete mycelial growth inhibition in all the test pathogens at a concentration of 7-8 μ l/ml after five days of incubation. The minimum inhibitory concentration and minimum fungicidal concentration of the essential oil on the test fungi were in the range of 7-8 μ l/ml and 8-10 μ l/ml, respectively. These findings confirm the fungicidal properties of *E. camaldulensis* essential oils and their potential use in the management of economically important *Fusarium spp.* And as possible alternatives to synthetic fungicides. (Martin and John., 2017).

The essential oil from *O. gratissimum* at 200 ppm significantly reduced the mycelial growth of *F. moniliforme*, *A. flavus* and *A. fumigatus* by 86 per cent, 66 per cent and 62 per cent, respectively. Total inhibition of conidial germination of these fungi was observed at 500, 600 and 800 ppm, respectively (Nguefack *et al.*, 2004).

Similarly from our study the eugenol at 0.05 per cent and 0.1 per cent significantly reduced the mycelial growth of *A. flavus* by 42 per cent and 48 per cent respectively and significantly reduced the mycelial growth of *R. bataticola* by 85 per cent at 0.05 per cent. Total inhibition of both fungi was observed at 0.5, 1 and 2 per cent.

Ajowain seed oil at 0.05 and 0.1 per cent significantly reduced the mycelial growth of *A. flavus* by 40 and 85 per cent respectively and significantly reduced the mycelial growth of *R. bataticola* by 84 per cent at 0.05 per cent. Total inhibition of both fungi was observed at 0.5, 1 and 2 per cent.

Rose oil at 0.05 and 0.1 per cent significantly reduced the mycelial growth of *A. flavus* by 46 and 89 per cent respectively and significantly reduced the mycelial growth of *R. bataticola* by 48 and 88 per cent at 0.05 and 0.1 per cent respectively. Total inhibition of both fungi was observed at 0.5, 1 and 2 per cent.

Clove oil at 0.05 and 0.1 per cent significantly reduced the mycelial growth of *A. flavus* by 46 and 89 per cent respectively and significantly reduced the mycelial growth of *R. bataticola* by 48 and 88 per cent at 0.05 and 0.1 per cent respectively. Total inhibition of both fungi was observed at 0.5, 1 and 2 per cent.

Cinnamon oil at 0.05 per cent significantly reduced the mycelial growth of *A. flavus* by 31 per cent and significantly reduced the mycelial growth of *R. bataticola* by 27 per cent at 0.05 and 0.1 per cent. Total inhibition of both fungi was observed at 0.25, 0.5, 1 and 2 per cent.

Evaluation of the antifungal properties of the plant extracts *in vitro*

The experiment was conducted under laboratory conditions where the twenty four plant extracts were diluted in three concentrations (0.5, 1 and 2 per cent) to determine the antifungal activity against *A. flavus* and *R. bataticola*. But the results obtained were insignificant.

Neem (*Azadiractha indica*) leaf extract was observed superior in all the concentrations in inhibiting the mycelial growth of the test fungus (*R. bataticola*) up to 144 hr which was superior over control and rest of the leaf extracts used during the course of investigation. Effectivity of neem (*Azadiracta indica*) leaf extract against *R. bataticola* was also demonstrated by Shivpuri *et al.*, (1997), Sindhan *et al.*, (1999), and Sharma *et al.*, (2005). The neem (*Azadiracta indica*) leaf extract contains azadirachtin and monoterpenes which show toxic effect against the *R. bataticola*. The plant extracts used in our study have not shown inhibitory activity and needs to screen more plant extracts to uncover the inhibitory molecules from the plant sources. The sources used plant extracts may not be

harboring the molecules for the inhibition of the fungal survival pathways. Hence, these plant extracts may be acting as nutrients for the fungal survival.

(Sindhani *et al.*, 1999) Plant leaf extracts were found toxic to the mycelial growth of *R. bataticola*, however the difference in their toxication varied Jatropha (*Jatropha curcas*) was observed to be inhibitory at 10% concentration within 48 hours and later its toxicity declined. Similarly, Datura (*Datura suaveolens*), Eucalyptus (*Eucalyptus globulus*), Aak (*Calotropis procera*), Tulsi (*Ocimum sanctum*), and Mehandi (*Lawsonia inermis*) were noted to be toxic to the mycelial growth but their efficacy declined as the time lapsed. However Jatropha (*Jatropha curcas*) was recorded to be effective in reducing the mycelial growth of the test fungus. The results were in accord with the findings of Sindhani *et al.* (1999). The effectiveness of these extracts may be due to the presence of antifungal constituents in the form of phenolic substances and resinous gummy and non-volatile substance of unknown nature (Skinner., 1995). Inhibition of mycelial growth of *R. bataticola* in this study may be attributed to the presence of antifungal properties and inhibitory compounds in the control of root rot diseases.

Use of botanicals for prevention of plant diseases without the mention of their deleterious effect has been recorded in ancient Indian literature. Nene (1996) reported several botanicals *viz.*, fruits of Brhati (*Solanum indicum* L.), Sandal (*Santalum album* L.) and Mahuva (*Madhuca indica* L.), seeds of Mehandi (*Lawsonia inermis* L.), Babool (*Acacia nilotica* L.) having antimicrobial activities against some plant disease causing organisms. Democritus (470 BC) tried plant extracts for controlling disease (Sharville, 1960). Since then there has been several recorded and unrecorded mention of their uses against a large number of microbial organisms. A number of angiospermic plants have been reported for the presence of antimicrobial activities by several workers (Nene, 1996; Khan, 1999, Sharma, 2000 and Roat, 2000). The preparation from several parts of plants such as seeds, leaves, flowers, fruits, roots, *etc.* have been used for the presence of antifungal activities.

Gupta *et al.* (1996) adopted poison food technique and reported that the leaf extract of *Pongamia pinnata* was highly fungitoxic to and *F. pallidorseum*, *F. moniliformae* inhibiting mycelial growth on plates by 78.2 and 84.3 per cent respectively. Similarly, *F. oxysporum* was checked by different partially purified plant products components in the present studies. Rodriguez and Valendia (1998) while working with *Colletotrichum* species

in vitro reported that extracts of clove (*Syzygium aromaticum*) inhibited the growth and sporulation of the fungus. The growth of *Colletotrichum gloeosporioides* from yam was inhibited in the present experiment by the use of different plant products components. In the present investigation clove oil tested was found effective in controlling the growth of *A. flavus* and *R. bataticola*.

Evaluation of the antifungal properties of the synthetic chemicals *in vitro*

The experiment was conducted under laboratory conditions where the sixty four synthetic chemicals were diluted in three concentrations (1, 10 and 100 ppm) to determine the antifungal activity against *A. flavus* and *R. bataticola*. The synthetic chemicals were found ineffective in controlling the fungal growth *in vitro* as these are lactone derived hence may not be useful as inhibitory molecules for the fungal growth inhibition.

Mode of action as antifungal activity of essential oil

The antifungal activity of essential oil might be caused by the properties of terpenes/terpenoids, due to their highly lipophilic nature and low molecular weight. These essential oils, works principally by blocking cell communication mechanisms, inhibiting fungal biofilm formation and inhibition of the mycotoxin production. These also disintegrate cell wall integrity, plasmolysis, altering anti-oxidant enzyme system, causing cell death or inhibiting the sporulation and germination of fungi (Nazzaro et al., 2017).

In the present study peroxidase activity recorded was ranged from 4.282 U/ml/min to 2.191 U/ml/min as the time period was increased from 4 hr to 24 hr in control. Similarly in eugenol treated it was found that the decrease from 3.1 U/ml/min to 1.644 U/ml/min, in ajowain seed oil treated it was found that the decrease from 7.093 U/ml/min to 3.368 U/ml/min, in rose oil treated it was found that the decrease from 6.544 U/ml/min to 4.018 U/ml/min, in clove oil treated it was found that the decrease from 3.128 U/ml/min to 1.762 U/ml/min and in cinnamon oil treated it was found that the decrease from 6.795 U/ml/min to 3.324 U/ml/min in *A. flavus*.

Similarly in *R. bataticola*, the peroxidase activity recorded was ranged from 16.931 U/ml/min to 7.084 U/ml/min when the time period was increased from 4 hr to 24 hr in control. In eugenol treated it was found that the decrease from 3.01 U/ml/min to 1.896 U/ml/min, in ajowain seed oil treated it was found that the decrease from 7.758 U/ml/min

to 3.527 U/ml/min, in rose oil treated it was found that the decrease from 4.64 U/ml/min to 3.759 U/ml/min, in clove oil treated it was found that the decrease from 4.683 U/ml/min to 3.049 U/ml/min and in cinnamon oil treated it was found that the decrease from 3.715 U/ml/min to 1.701 U/ml/min.

The results were inconformity as Tian *et al.*, 2012 assessed the activities of the mitochondrial dehydrogenases exposed to dill oil were detected by XTT, which metabolically decreases by mitochondrial dehydrogenases in viable cells. The observations revealed that activities of the mitochondrial dehydrogenases are evidently inhibited by dill oil, which may interfere with the citric acid cycle and inhibit the synthesis of ATP in the mitochondria of *A. flavus*. Exposure to dill oil resulted in an elevation of MMP, and in the suppression of the glucose-induced decrease in external pH at 4 μ l/ml. Decreased ATPase and dehydrogenase activities in *A. flavus* cells were also observed in a dose-dependent manner. The above dysfunctions of the mitochondria caused ROS accumulation in *A. flavus*. A reduction in cell viability was prevented through the addition of L-cysteine, which indicates that ROS is an important mediator of the antifungal action of dill oil. In summary, the antifungal activity of dill oil results from its ability to disrupt the permeability barrier of the plasma membrane and from the mitochondrial dysfunction-induced ROS accumulation in *A. flavus*.

A. flavus produces aflatoxin in response to oxidative stress (Kim *et al.*, 2005) and the inclusion of phenolic antioxidants in media prevents its accumulation (Chiple and Uraih 1980; Kim *et al.*, 2006). Recent reports show that application of abscisic acid, a mediator of plant abiotic stress or H₂O₂ to maize leaves activates a mitogen-activated protein kinase, which enhanced the expression of antioxidant genes, including Cat1 (Zhang *et al.*, 2006). In addition, catalase activity was increased. Similarly in the present study on exposure to essential oil increases the catalase activity. The increase in catalase activity recorded was ranged from 0.247 U/ml/min to 0.626 U/ml/min when the time period was increased from 4 hr to 24 hr in control. Similarly in eugenol treated it was found that the decrease from 0.142 U/ml/min to 0.482 U/ml/min, in ajowain seed oil treated it was found that the decrease from 0.362 U/ml/min to 0.671 U/ml/min, in rose oil treated it was found that the decrease from 0.281 U/ml/min to 0.595 U/ml/min, in clove oil treated it was found that the decrease from 0.062 U/ml/min to 0.151 U/ml/min and in cinnamon oil treated it was found that the decrease from 0.182 U/ml/min to 0.280 U/ml/min in *A. flavus*.

Similarly in *R. bataticola*, the increase in catalase activity recorded was ranged from 0.247 U/ml/min to 0.626 U/ml/min when the time period was increased from 4 hr to 24 hr in control. Similarly in eugenol treated it was found that the decrease from 0.142 U/ml/min to 0.482 U/ml/min, in ajowain seed oil treated it was found that the decrease from 0.362 U/ml/min to 0.671 U/ml/min, in rose oil treated it was found that the decrease from 0.281 U/ml/min to 0.595 U/ml/min, in clove oil treated it was found that the decrease from 0.062 U/ml/min to 0.151 U/ml/min and in cinnamon oil treated it was found that the decrease from 0.182 U/ml/min to 0.280 U/ml/min.

VI. SUMMARY AND CONCLUSIONS

Twelve essential oils namely, orange oil, peppermint oil, citronella oil, methyl eugenol, eugenol, ajowain seed oil, cumin seed oil, rose oil, lavender oil, clove oil, cinnamon oil and eucalyptus oil, 24 plant extracts and 64 lactone based synthetic chemicals were evaluated against *A. flavus* and *R. bataticola*.

Essential oils tested in the present investigation were found effective but plant extracts and chemical entities tested were not found effective in controlling the *A. flavus* and *R. bataticola*.

In poison food technique, all the essential oils significantly inhibited the growth of *A. flavus* and *R. bataticola*. But the utmost inhibition of *A. flavus* colony growth was recorded with clove oil followed by rose oil, cinnamon oil, ajowain seed oil and eugenol. The other essential oils were effective and growth inhibition was recorded with citronella oil, peppermint oil, methyl eugenol and cumin seed oil at higher concentrations (1 and 2 per cent). The remaining essential oils were not so effective and minimum growth inhibition was recorded with lavender oil, eucalyptus oil and orange oil at higher concentration (2 per cent). Similarly, the highest inhibition of *R. bataticola* growth was recorded with clove oil followed by eugenol, ajowain seed oil, rose oil and cinnamon oil. The other essential oils were effective and growth inhibition was recorded with methyl eugenol, cumin seed oil, peppermint oil and citronella oil at higher concentrations (1 & 2 per cent). The remaining essential oils were not so effective and minimum growth inhibition was recorded with lavender oil, eucalyptus oil and orange oil at higher concentration (2 per cent).

It is concluded that the essential oils were effective in managing *A. flavus* and *R. bataticola* and if due emphasis is given to them, they can gradually replace the synthetic or chemical fungicides easily. These plant product components may be environmentally non pollutive in nature and negate the possibility of development of fungicide resistant strains of plant pathogenic fungi.

Further study and recommendations

Field experiments should be considered in order to gain information about the effectiveness of the essential oils tested for fungal control in crops. The result will be very important since the field studies will reveal efficacy of essential oils in controlling fungi as they interact with environmental factors. The tests could be conducted also on a wide range of pathogens, so that in vivo assessments can be broadened.

VII. REFERENCES

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