Research Journal of Recent Sciences _____ ISSN 2277-2502
Vol. **1(9),** 67-73, September **(2012)** *Res.J.Recent Sci.*

In vitro Antifungal activities of Essential oils extracted from Fresh Leaves of *Cinnamomum zeylanicum* and *Ocimum gratissimum* against Foodborne pathogens for their use as Traditional Cheese Wagashi conservatives

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Available online at: www.isca.in
Received 18th July August 2012, revised 25th July 2012, accepted 30th July 2012

Abstract

Alternative natural additives are needed in order to guarantee food safety in preservation against foodborne pathogens. Aromatic plants are traditionally employed for seasoning and prolongation of shelf life of food. The majority of their properties are due to the essential oils produced by their secondary metabolism. The objective of this study was to assess in vitro antifungal activity of essential oils extracted from fresh leaves of Cinnamomum zeylanicum and Ocimum gratissimum obtained by hydrodistillation with Clevenger apparatus against spoilage and pathogens moulds Aspergillus terreus, Aspergillus ustus, Aspergillus niger, Aspergillus aculeatus, Penicillium brevicompactum and Scopulariopsis brevicaulis both isolated from wagashi. The screening of their antifungal activity was carried out by determination of antifungal activity parameters as mycelial growth inhibition, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Results obtained from this study showed that Ocimum gratissimum essential oil was the most effective as antifungal agent among the essential oils tested due probably to its prominent concentration in phenolic compound thymol with MIC ranged from 600 to 800 mg/L. Scopulariopsis brevicaulis and Aspergillus terreus were the most sensible strains with minimum fungicidal concentration (MFC) varying from 600 to 1000 mg/L while Aspergillus aculeatus was the most resistant mould of all to essential oil of cinnamon. With the rise of this study, it is shown that Cinnamomum zeylanicum and especially Ocimum gratissimum essential oils could be regarded as a very promising preservative for wagashi in order to prevent the mycelia growth responsible of its deterioration.

Keywords: *Cinnamomum zeylanicum*, *Ocimum gratissimum,* essential oils, wagashi, Benin, antifungal activity.

Introduction

The microbiological quality of a food constitutes is one of the essential bases of its aptitude to satisfy the safety of the consumer. A food, exposed to deterioration by the fungus can have a decreasing in its sensory, nutritive and medical characteristics. Fungi are also responsible for the formation of taste and the production of allergenic compounds and mycotoxins[1]. Traditional cheese locally called wagashi obtained without ripening in Benin and often colored by red sorghum[2] is an important source of animal proteins. Due to its proteins content, wagashi could efficacy contribute to the resolution of nutritional problems due to the deficiency of proteins[3]. However, wagashi is produced and preserved using rudimentary methods under unsanitary conditions which may lead to the contamination of the product by toxinogenic or pathogenic microorganisms especially fungi[4]. The contamination of this product by fungi may contribute to the loss of its quality and safety. In fact, the fungal growth may result in several kinds of cheese spoilage: off-flavours, toxins, mycolytic enzymes and rotting[5]. Furthermore, fungi produce allergenic compounds and toxic metabolites which may penetrate the cheese and affect the consumer's health[6,7]. A better control measures to prevent spoilage of wagashi is necessary to avoid its contamination by mycoflora and minimize public health hazards. The use of synthetic fungicides to control cheese spoilage moulds has been discouraged due to their effects on cheese, carcinogenicity, teratogenicity, high and acute residual toxicity, long-term degradation[8]. One of the major problems related to the use of these chemicals is that the fungi develop resistance[9,10]. The use of higher concentrations of chemicals, to overcome the microbial resistance further enhances the risk of high level toxic residues in the products[11]. Alternative natural additives are therefore needed in order, to guarantee food safety in preserved wagashi. Aromatic plants are traditionally employed for seasoning and prolongation of shelf life of food. The majority of their properties are due to the essential oils produced by their secondary metabolism[12]. Essential oils (EOs) as antimicrobial agents are recognized as safe natural substances to their user and for the environment and they have been considered at low risk for resistance development by pathogenic microorganisms[13]. Among the aromatic plants, *Ocimum gratissimum* and *Cinnamomum zeylanicum* used as a food spices had shown strong antimicrobial activitie[14,15,16,17,18]. Their efficacy on cheese

mycoflora has been few studied. The efficacy of these essential oils on several moulds isolated from wagashi must be verified in order to measure their potential biopreservation for the valorization of this product. According to our studies, the main components of *Ocimum gratissimum* essential oil were thymol, γ-terpinene and p-cymene while that of *Cinnamomum zeylanicum* essential oil were cynnamyl acetate, cinnamaldehyde and benzyl benzoate[19,20]. The objective of this research was to assess *in vitro* antifungal activity of essential oils of *Cinnamomum zeylanicum* and *Ocimum gratissimum* against six moulds, *Aspergillus terreus*, *Aspergillus ustus*, *Aspergillus niger, Aspergillus aculeatus, Penicillium brevicompactum* and *Scopulariopsis brevicaulis* both isolated from wagashi produced in Benin for their potential use as wagashi biopreservatives.

Material and Methods

Plants material and extraction of the essential oils: Fresh leaves of *Ocimum gratissimum* and *Cinnamomum zeylanicum* were collected in Abomey-Calavi area (06°27'0.00 N and 2°21'0.00'' E) at University of Abomey-Calavi in Republic of Benin at October-November 2011 and were identified by Dr Yedomohan of National Herbarium of Benin. They were hydrodistilled for about 3 hours, using a Clevenger apparatus. Oil recovered in a dark sterile glass was dried over anhydrous sodium sulfate and stored at +4 °C until it was used[21].

Strains of filamentous fungi tested: The fungi used in this study were: *Aspergillus terreus*, *Aspergillus ustus*, *Aspergillus niger, Aspergillus aculeatus, Penicillium brevicompactum* and *Scopulariopsis brevicaulis*. They have been isolated and identifying from a traditional cheese wagashi collected near its vendors. Colonies of these moulds isolated from DBRC medium [Rose-Bengal Chloramphenicol Agar Base (CM0549 Oxoid, LTD Basingstoke, Hampshire, England), Chloramphenicol supplement (SR0078E, Oxoid), Dichloran (FLUKA Analytical Lot # SZB8239XV Sigma-ALORICH Product of Germany)] by dilution method[22] were purified by streaking onto Malt Extract Agar and then three point inoculated onto MEA (LAB 37, United Kingdom) and Czapeck Yeast autolysate Agar [Czapeck-Dox Liquid medium modified (CM 0095 Oxoid LTD Basingstoke, Hampshire, England), $NaNO_3$ (Qualikems, Laboratory reagent Product N° S061112), KCl (Qualikems, Laboratory reagent Product N°P021112), $MgS0_4.7H_20$ (Qualikems, Laboratory reagent Product N°M009112), Yeast extract (LP 0021 Oxoid), $KH2PO_4$ (Pro analysi, Merk Darmstadt Germany A830673), Sucrose (AnalaR Lot K26990786 009 BDH Laboratory supplies, England), Agar bacteriological (LP 001 Oxoid)] before identification based both on macroscopic characters (colony growth, colony diameter) and microscopic characters using the identification schema of Samson et al.[23] and Pitt and Hocking[24].

Antifungal assay: The test was performed by the agar medium assay[25]. Potato dextrose agar (PDA) medium with different concentrations of essential oil (200, 400, 600, 800 or 1000 $mg.L^{-1}$) were prepared by adding appropriate quantity of essential oil to melted medium, followed by addition of Tween 80 (100 µL to 100 mL of medium) to disperse the oil in the medium. About 20 ml of the medium were poured into glass Petri-dishes (9 cm x 1.5 cm). Each Petri-dish was inoculated at the centre with a mycelial disc (6 mm diameter) taken at the periphery of a fungus colony grown on PDA for 48 h. Positive control (without essential oil) plates were inoculated following the same procedure. Plates were incubated at 25°C for 8 days and the colony diameter was recorded each day. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of essential oil in which no growth occurred. The MGI (Mycelia Growth Inhibition) percentage was calculated according to the equation:

$$MGI = \frac{dc-dt}{dc} \text{X}100$$

where dc = mean diameter for control – 6 mm and dt = mean diameter for treated mycelium – 6 mm.

The minimal fungicidal concentration (MFC) values were determined by the method described by Angelini et al.[13]. This was done by subculturing the inhibited fungal discs at MICs on PDA medium without essential oil. Observations were recorded after 7 days of incubation at 25°C. Fungal growth on the seventh day was indicative of a fungistatic nature, while the absence of fungal growth denoted a fungicidal action of the oil.

Statistical analysis: Data from three independent replicate trials were subjected to statistical analysis using statistica version 6.0[26]. Differences between means were tested using Z-test.

Results and Discussion

Recently, the scientific interest in biological properties of essential oil (EO) has been increased. New researches about biological active secondary compounds present in EO of plants have been seen as a potential way to control fungal contamination. Our study had assessed potential antifungal activity of essential oils *Cinnamomum zeylanicum* and *Ocimum gratissimum*. The MGI, fungistatic and fungicidal activities values of these two essential oils against the tested fungi are reported in table-1. The results showed that the percentages of mycelial growth inhibition are significantly ($p < 0.05$) influenced by incubation time and essential oils concentrations. According to figures-1 and 2, mycelia growth was reduced with increasing concentration of essential oil while their growth increased with incubation time. Essential oil of *Ocimum gratissimum* had significant fungistatic activity against all the species investigated with MIC values ranged from 400 to 1000 mg/L and fungicidal activity against *Aspergillus terreus* (MFC= 1000 mg/L) and *Scopulariopsis brevicaulis* (MFC = 600 mg/L) whereas that *Cinnamomum zeylanicum* had total inhibited effect only on *Aspergillus terreus* (MIC= 400 mg/L and MFC = 600 mg/L), *Scopulariopsis brevicaulis* (MIC= 600 mg/L and MFC = 800 mg/L) and *Penicillium brevicompactum* (MIC= 1000

mg/L). *Aspergillus aculeatus* was the most resistant strain to *Cinnamomum zeylanicum* essential oil with MGI equal to 59.52% whereas *Aspergillus terreus* and *Scopulariopsis brevicaulis* were the most sensible on which the two volatiles extracts tested had fungicidal activity with MFC ranged from 600 to 1000 mg/L. Essential oil of *Ocimum gratissimum* was the most promising extract as natural agent for biopreservation of wagashi of the two essentials oils. In fact, essential oil of clove basil oil had at least fungistatic activity on all species tested whereas that of cinnamon didn't totally inhibit some of species tested such as *Aspergillus aculeatus, A. niger* and *A. ustus*. The antifungal activity expressed by the two oils on species tested is thought to depend on specific toxicity of their single main active constituents or by their synergic effect[27]. Furthermore, the biological activity of these oils is probably due their prominent concentration in thymol for *Ocimum gratissimum* and cinnamaldehyde for *Cinnamomum zeylanicum*.

The more interesting fungistatic activity of essential oil of clove basil oil than that of cinnamon oil is in relation to its richness in phenolic compound, the thymol. Generally, the essential oils possessing the strongest antimicrobial properties against foodborne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol[28]. An important characteristic of thymol is its hydrophobicity, which enables it to partition in the lipids of the fungal cell membrane, disturbing the structures and rendering it more permeable and leakage of ions and other cell contents can then occur[29-31].

Many studies have assessed antifungal activities of essential oil of *Ocimum gratissimum* against different foodborne pathogens. It was reported that volatile oil of *O. gratissimum* has been reported to have significant antimicrobial effects against both fungi and bacteria[32]. Prakash et al.[33] have reported that clove basil oil can be used as antimicrobial agent against fungal and aflatoxin B_1 contamination of spices. According to Nguefack et al.[34], *Ocimum gratissimum* essential oil detains good antifungal properties against mycotoxinogenic fungi. Essential oil *Ocimum gratissimum* studied by Faria et al.[35] have showed that essential oil of clove basil is active against phytopathogenic fungi.

The less antifungal activity of cinnamon oil compared to *Ocimum gratissimum* essential oil could be due to its high content in cinnamyl acetate and benzyl benzoate which are no reported to possess antifungal activity in literature to our knowledge. Therefore, it becomes evident that there is a relationship between the average presence of cinnamaldehyde in essential oil of cinnamon and the moderate antifungal activity of this oil. Indeed, Simic et al.[17] have reported that cinnamon oil had antifungal activity on many fungal species in relation to its content of cinnamaldehyde as major agent of inhibition of fungal growth. Ooi et al.[36], Shahverdi et al.[37] and Unlu et al.[18] have also confirmed this activity of cinamaldehyde, and could justify this activity against fungi recorded due to the possession of their polar hydrophilic functions (OH, COOH, NH_2, and NO_2) according to Diallo[38]. Our study concerned cinnamon oil is contrary to those of literature where cinnamon oil possessed high antifungal activity[16,17,34,35]. This difference of reaction of cinnamon oils could be due to their differences in chemical composition.

Conclusion

The present work has assessed and compared the antifungal activities of two essentials oils extracted from *Ocimum gratissimum* and *Cinnamomum zeylanicum* against fungi isolated from traditional cheese wagashi. Results obtained indicate that the essential oils of *Ocimum gratissimum* and *Cinnamomum zeylanicum* have an interesting antifungal activity. Meanwhile, essential of *Ocimum gratissimum* was the most active against all the tested strains. These oils especially *Ocimum gratissimum* essential oil could be used as natural antimicrobial agent in the fight against moulds species responsible for wagashi contamination. For the practical use of these oils as novel fungal-control agents of wagashi, further research is needed on safety issues for human health and this product acceptability when treated with clove basil oil.

Acknowledgements

The authors acknowledge University Council of Development (CUD) for their financial support. They thank Professor Menut Chantal of University of Clemont-Ferrand in France, Professor Dossevi Lordson, Dr Noudogbessi Jean Pierre and Mr. Dossa François for their technical help.

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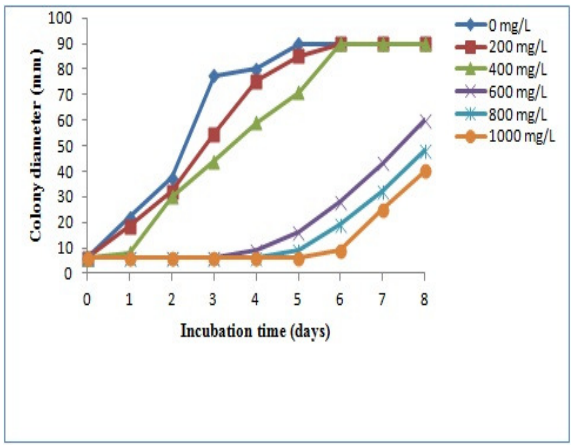
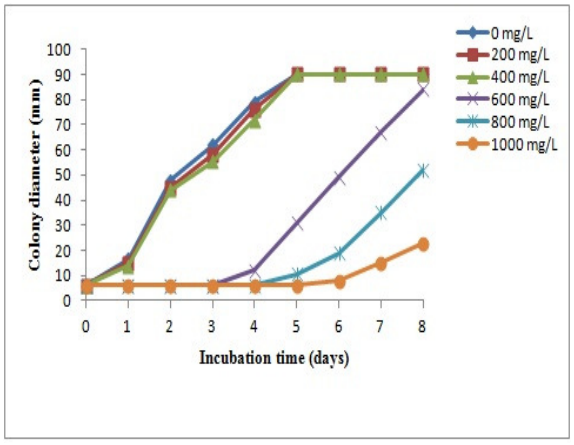
Table-1
Mycelial growth inhibition, fungistatic and fungicidal activity of essential oils of *Cinnamomum zeylanicum and Ocimum gratissimum* on tested fungi

Essential oil (mg/L)	Mycelia growth inhibition (%)					
	A. aculeatus	***A. niger***	***A. terreus***	***A. ustus***	***P. brevicompactum***	***S. brevicaulis***
C. zeylanicum						
200	0	0	9.58 ± 0.90b	0	57.69 ± 0.23a	0
400	0	0	100a (FS)	0	84.61 ± 0.54b	69.04 ± 1.72c
600	35.71 ± 0.43c	7.14 ± 0.28e	100a (FS) (FC)	17.85 ± 0.71d	92.30 ± 0.76b	100a (FS)
800	50.00 ± 0.20d	45.23 ± 0.81e	100a (FS) (FC)	53,57 ± 0.14c	96.15± 0.38b	100a (FS) (FC)
1000	59.52 ± 0.81c	79.76 ± 1.90b	100a (FS) (FC)	79,76± 1.79b	100a (FS)	100a (FS) (FC)
O. gratissimum						
200	14.28 ± 0.57c	0	42.46 ± 2.05a	0	30.76 ± 0.92b	29.76 ± 1.94b
400	59.52± 0. 38d	57.14± 0.85e	80.82 ± 1.19b	16.07 ± 0. 14f	67.94 ± 0.87c	100a (FS)
600	96.42± 1.55b	85.714 ± 2.8d	90.41 ± 1.36d	92.85 ± 0.71c	79,48 ± 1.42e	100a (FS) (FC)
800	100a (FS)	95.23± 0.93b	100a (FS)	98.80 ± 0.59a	94,87 ± 0.25b	100a (FS) (FC)
1000	100a (FS)	100a (FS)	100a (Fs) (FC)	100a (FS)	100a (FS)	100a (FS) (FC)

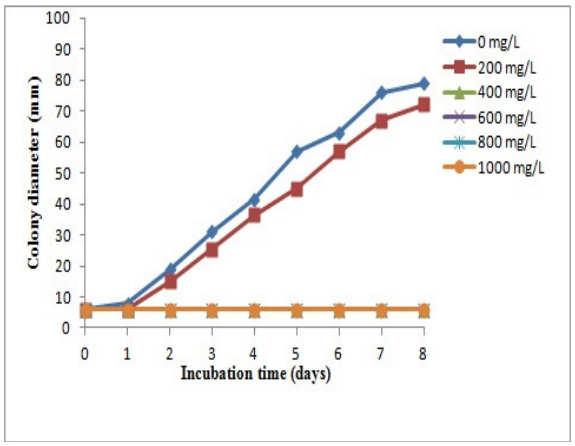
FS: fungistatic activity; FC: Fungicidal activity; Data in the line followed by different letters are significantly different ($p < 0.05$). The values are means of three repetitions ± standard deviation



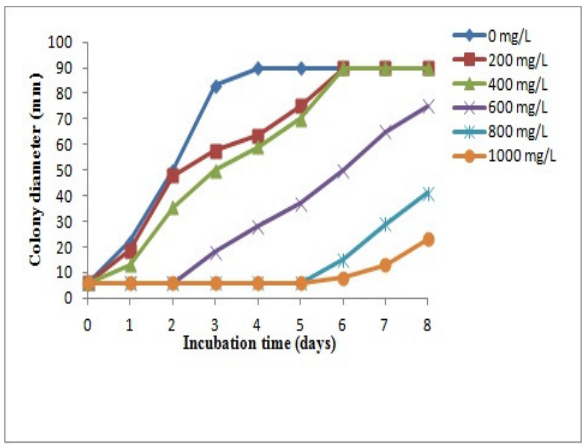
a) Effect of different concentrations of *Cinnamomum zeylanicum essential* oil on *Aspergillus aculeatus* **growth**



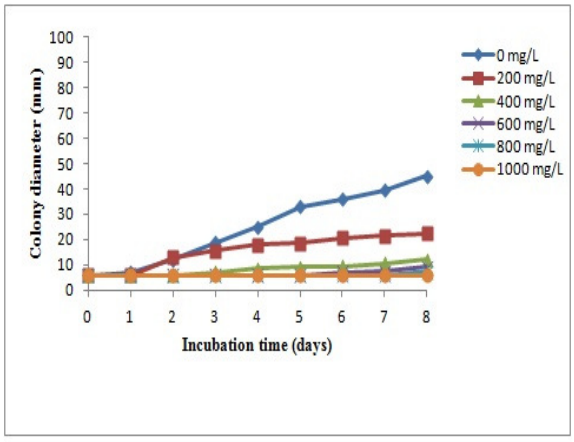
b) Effect of different concentrations of *Cinnamomum zeylanicum essential* oil on *Aspergillus niger* growth



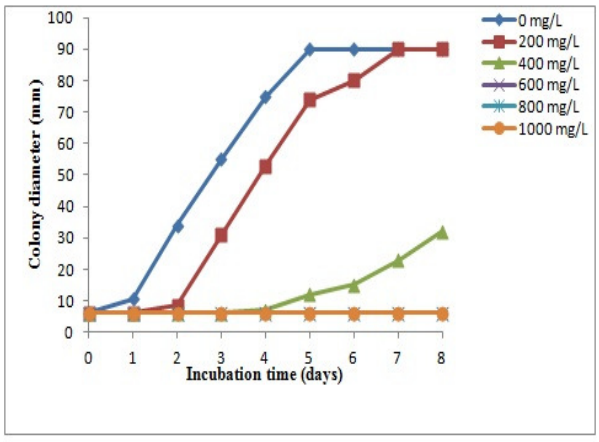
c) Effect of different concentrations of *Cinnamomum zeylanicum essential* oil on *Aspergillus terreus* growth



d) Effect of different concentrations of *Cinnamomum zeylanicum essential* oil on *Aspergillus ustus* growth

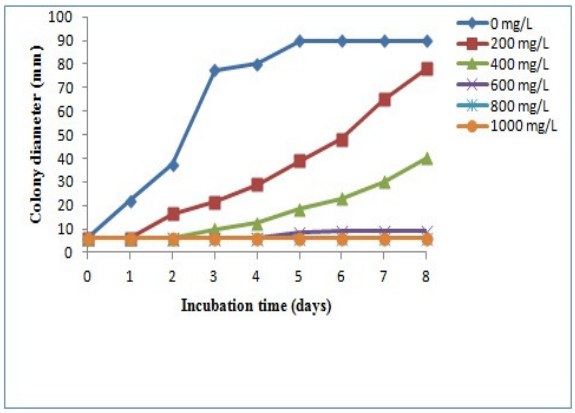


e) Effect of different concentrations of *Cinnamomum zeylanicum essential* oil on *Penicillium brevicompactum* growth

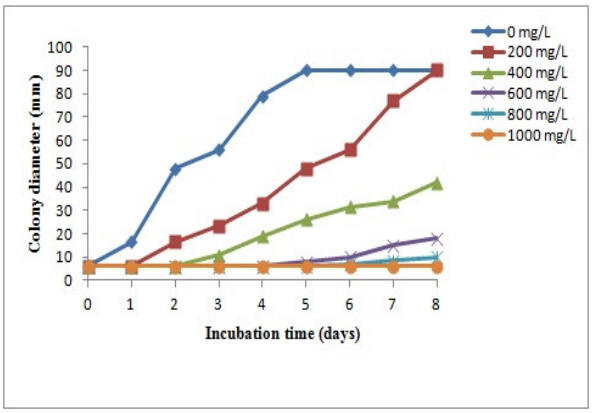


f) Effect of different concentrations of *Cinnamomum zeylanicum essential* oil on *Scopulariopsis brevicaulis* growth

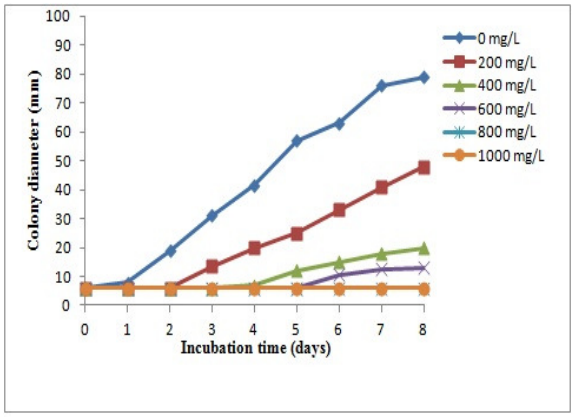
Figure-1
Effect of different concentrations of essential oil of *Cinnamomum zeylanicum* against moulds investigated



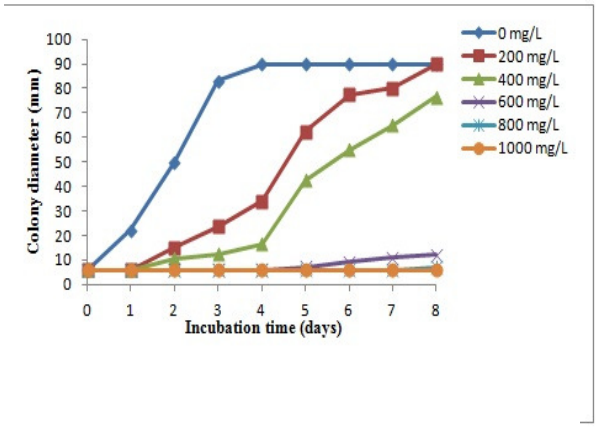
a') Effect of different concentrations of *Ocimum gratissimum essential* oil on *Aspergillus aculeatus*



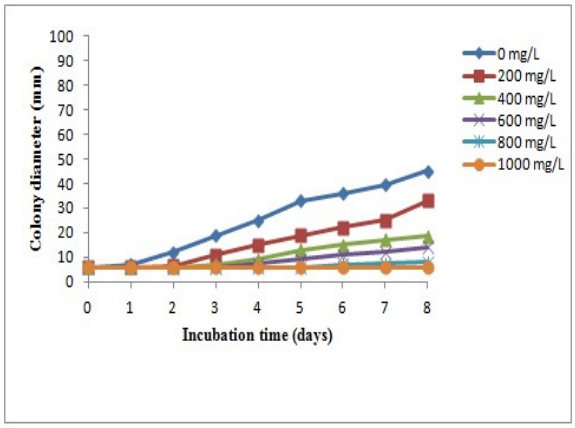
b') Effect of different concentrations of *Ocimum gratissimum essential* oil on *Aspergillus niger* growth



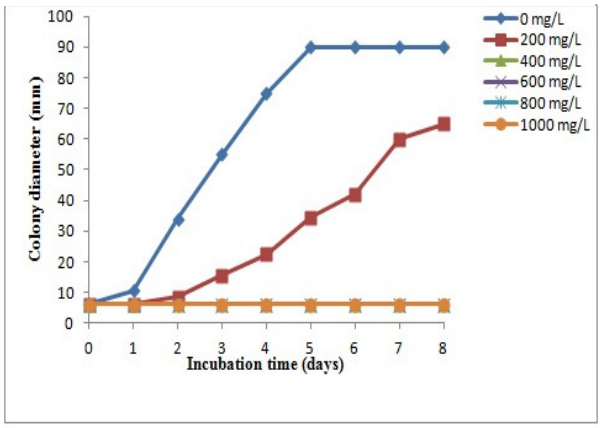
c') Effect of different concentrations of *Ocimum gratissimum essential* oil on *Aspergillus terreus* growth



d') Effect of different concentrations of *Ocimum gratissimum essential* oil on *Aspergillus ustus* growth



e') Effect of different concentrations of *Ocimum gratissimum essential* oil on *Penicillium brevicompactum* growth



f') Effect of different concentrations of *Ocimum gratissimum essential* oil on *Scopulariopsis brevicaulis* growth

Figure-2
Effect of different concentrations of essential oil of *Ocimum gratissimum* against moulds investigated