International Journal of Applied and Pure Science and Agriculture
ISSN Online - 2394-5532 Impact Factor : 4.446 by SJIF ISSN Print - 2394-823X

SCREENING EFFECTIVE AND PERSISTING ESSENTIAL OILS AGAINST FUNGI DETORIATING PALM LEAF PRODUCTS

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Abstract

The potential effect of clove, cinnamon, mustard, peppermint, eucalyptus, citronella, camphor, rose, lemon and lemon grass essential oils against fungi identified from Areca palm leaves and Palmyra palm leaf sheath were investigated. The fungi were identified by 18s rRNA sequencing method. An agar dilution method was employed to determine the minimum inhibitory concentration (MIC) of essential oils. Zone inhibition tests and the inhibitory effect of the leaf and sheath dip treated and vapour treated with essential oils against those fungi were examined. With an MIC of 0.02 µl ml^{-1} mustard essential oil had the strongest inhibitory effect. The efficacy of mustard oil vapour on the molds inhibition was relatively higher when compared to the liquid phase. It is also comparatively more potential than chemical fungicides.

Keywords: Palmyra palm, Areca palm, Fungicides, Essential oil, 18srRNA, MIC, Zone inhibition test

I. INTRODUCTION

In southeast Asia and India, areca palm (*Areca catechu*) leaf sheath dropped naturally and Palmyra palm (*Borassus flabellifer*) leaf have been traditionally used as an environmentally friendly food serving and packaging material for centuries (Kalita *et al.,* 2008). Now a day with modern technology, this natural and rigid material is compressed into different shapes and used to serve and pack food. Over the past 20 years, the use of bio-based packaging materials to prolong the shelf-life and improve the quality of fresh food products has been receiving increased attention (Del Nobile *et al.*, 2009). Palmyra palm leaves are employed in making utilitarian, aesthetic, artistic, creative, culturally attached, decorative, functional, traditional, religiously and socially symbolic and significant handicraft products too.

The presence and growth of fungi in the above materials may cause food spoilage and also results in a reduction in quality and quantity (Omidbeygi *et al.,* 2007). Aflatoxins produced by *Aspergillus* species are known to be potent hepato carcinogens in animals and humans. Therefore, the presence of toxigenic fungi and mycotoxins in foods packing and serving materials and handicraft products present a potential hazard to human and animal health.

Some chemical compounds are used as fungicides and they are commonly made up of 90% sulfur and are very toxic. Copper sulfate (bluestone) is one of the commonly used fungicides among copper forms. Bordeaux mixture a combination of copper sulfate and lime is an oldest fungicide that successfully used for more than 150 years on ornamentals, fruits and vegetables. Phosphorous acid another effective fungicide that acts over the fungi either by inhibiting a particular process or by inducing a defence response in the agent to show inhibiting activity. Biphenyl is also a fungicide that has the ability to inhibit the sporulation process of fungi.

As the chemical preservatives are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity, consumers tend to be mistrustful of chemical additives and thus there is demand for natural and socially more acceptable preservatives (Skandamis et al., 2001). An extensive significance has emerging on the use of essential oils to retard growth and mycotoxin production. A renewed interest in natural preservation appears to be stimulated by present food safety concerns, growing problems with microbial resistance, and a rise in production of minimally processed food, together with green image policies of food industries. Abundant reports have documented the antifungal effects (Aligiannis et al., 2001; Thompson, 1989) of plant essential oils. The cellular effects of aflatoxins may regulate by natural products and evidence suggests that aromatic organic compounds of spices can control the production of aflatoxins (Chatterjee, 1990). The use of the polymerase chain reaction (PCR) for microbial identification offers great advantage compared with conventional microbiological testing (Ferrer *et al.,* 2001; Makimura *et al.,* 1994; Rickerts *et al.,* 2007). Fungal primers specific for the conserved sequence of 18s rRNA gene common to all fungi have been used to detect fungal specimens (Anand et al., 2011).

The objective of this work is to evaluate the antifungal activity of ten essential oils namely clove oil, cinnamon oil, mustard oil, peppermint oil, eucalyptus oil, citronella oil, camphor oil, rose oil, lemon oil and lemon grass oil on fungi commonly found on the areca palm leaf sheath and palmyra palm leaf surface and compare them with the effect of chemical fungicides.

II. MATERIALS AND METHODS

Essential oil and leaf sheath

Food grade essential oils (clove oil, cinnamon oil, mustard oil, peppermint oil, eucalyptus oil, citronella oil, camphor oil, rose oil, lemon oil, and lemongrass oil) derived from steam distillation from Eastern Distributors, Coimbatore. The *Areca palm* leaf sheath and *Palmyra palm* leaves used for making handicraft products were obtained from Natural Fibre Handicraft centre, Punnayadi village, Kanyakumari district, Tamil Nadu, India.

Cultures

Three fungal strains were separated from *Areca palm* leaf sheath and *Palmyra Palm* leaf surfaces using Agar Plate technique in Potato Dextrose Agar (PDA) medium and morphological identification was carried out. The isolated fungi were the major causes of deterioration the leaf sheath, leaf and intermediate moisture food wrapped in them.

Preparation of Inoculam

Spores of the test fungi were obtained from mycelia grown on Potato Dextrose Agar (PDA) at 30°c for 14 days, and were collected by flooding the surface of the plates with ~5ml sterile saline solution (NaCl,8.5g/l water) containing Tween 80 (0.1%v/v). After the spores were counted using a haemocytometer, the suspension was standardized to concentrations of 10^7 spores / ml by dilution made using sterile water before use. The viability of all strains was checked using quantitative colony counts at 10^7 CFU/ml.

Fungal identification

DNA was extracted from the fungal pellet and purified using PureFast® Fungal Genomic DNA purification kit. Fungal ITS forward and reverse primers were used in the PCR reaction Each 50 ml PCR reaction consisted of 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives. PCR reactions were run using the following parameters: (1) 94ºC for 3 min, (2) 30cycles of 94º C for 1 min, 58º C for 1 min, and 72º C for 1min, and (3) 72º C for 5 min. Clean PCR products were sequenced using ITS primers, assembled and edited. Nucleotide sequences were compared to those in the Gene Bank Database with the Basic Local Alignment Search Tool (BLAST)

algorithm to identify known closely related sequences. The 18s rDNA sequences were aligned and phylogenetic tree was constructed by neighbour joining method.

Inhibition of molds by Essential oils

Determination of minimal inhibitory concentration (MIC) of the essential oils on each test fungus was performed by the agar dilution method. The essential oil was added aseptically to sterile PDA to make an agar solution with essential oil the concentration used was 50 µl. The resulting agar solutions were immediately poured into petriplate dishes after vortex. The plates were spot inoculated with 100µl (10^7 spore/ml) of each fungus. The vegetable oil (olive oil) was used as a control. The inoculated plates were incubated at 25° C for 3 days. At the end of the incubation period, the plates were evaluated for the presence or absence of microbial growth. The MIC value was determined as the lowest concentration of the essential oil at which absence of growth was recorded (Narumol Matan *et al*., 2011).

Zone of Inhibition of leaf sheath Dip-Treated with Essential oils

The disc diffusion method was employed to determine a zone of inhibition (ZI) of *Areca palm* leaf sheath and *Palmyra palm* leaf discs treated with essential oils. One hundred micro litres of a suspension containing 10^7 CFU/ml of individual fungi was spread on the PDA plate. Sets of 3 random triplicate specimens of the leaf sheath disc of 6mm in diameter were dip-treated according to ASTM test method D4445-91 (American Society Testing Materials 1998) for 15s with each essential oil at its MIC obtained from inhibition of fungi tests. Vegetable oil (olive oil) was used as a control. Different dilutions of essential oils were made with methanol. The disc was placed in sterile beaker that was covered tightly with a plastic sheet to prevent drying of the essential oil and stored in an aseptic cabinet for 24h. This allowed the draining of excess oil and time for the essential oil to penetrate into the leaf sheath before inoculation of the PDA. The inoculated plates were then incubated at 25°C for 3 days. The antifungal activity of the treated leaf sheath was evaluated by measuring the zone of inhibition (the width in mm, of the clear zone outside the disc) against the test organisms (Narumol Matan *et al*., 2011).

Mold Test on *Areca palm* leaf sheath and *Palmyra palm* leaf using Mustard oil

Sets of 3 triplicate specimens of all the 3 leaf sheath plate of 10mm in width and 70mm in length were dip-treated with mustard oil over the range of 1-10 µg/ml. Vegetable oil (olive oil) was used as a control. Essential oils and vegetable oil were diluted with methanol. Dip-treated specimens were held in a closed container overnight at room temperature before inoculation with spores of the test fungus.

The dip-treated specimens were inoculated with 1ml of each spore inoculums (10^7 spores/ ml) and were incubated at 25° C with 100%RH chamber for 45 days. The specimens were then individually rated for fungus growth cover. The specimens were then individually rated for fungus growth cover on a 0-5 scale (0, no growth; 1, 20% cover; 2, 40% cover; 3, 60% cover; 4, 80% cover; 5, 100% cover) according to ASTM test method D4445-91 (American Society for Testing and Materials, West Conshohocken, 1998). The percentage area of stain and fungus (based on a control) for each essential oil concentration was calculated as (A/B) x100, where A is the total score for each fungus at each concentration of essential oil, and B is the total score for each fungus in the controls (Narumol Matan *et al*., 2011).

Zone of inhibition using vapour phase of essential oil

For investigation of the effect of essential oil vapour 1ml of each fungal spore suspension of 10^7 spores/ml was inoculated on a potato dextrose agar and incubated at 25° C for 12 hrs for getting the exponential growth phase of the fungi. Then 20µl of each essential oil was put on the sterile discs (6mm) in upper lid of the inverted petriplates where it gets converted to essential oil vapour. The petriplate was sealed with parafilm and further incubated for another 3 days at 25°C. Vegetable oil was used as the control. This allows the essential oil vapours to act against the fungi. Zone of inhibition was observed.

Inhibition of fungi by Fungicides

Determination of minimal inhibitory concentration (MIC) of the fungicides on each fungus was performed by the agar dilution method. The fungicides were added aseptically to sterile PDA. The resulting agar solution was immediately poured into petriplate dishes after vortex. The plates were inoculated with 100µl (10^7 spores/ml) of each spore inoculum. The plate that was not inoculated with the test fungi was used as a control. The inoculated plates were incubated at 25°C for 3 days. At the end of the incubation period, the plates were evaluated for the presence or absence of fungus growth. The MIC value was determined as the lowest concentration of the fungicides at which absence of growth of the fungi were observed.

Zone of inhibition of fungicides against fungi

Spore suspension of each fungus at the concentration of 10^7spores/ml was inoculated on PDA plates by spread plate technique. Discs dipped in selected fungicides ($CuSO_4$, OPA and Biphenyl) were prepared and placed onto the inoculated PDA plates, and incubated at 25° C for 3 days. After incubation zone of inhibition was measured and tabulated.

III. RESULTS AND DISCUSSION

Fungal identification

Based on the results of the 18s rRNA sequence analyses (BLAST and phylogenies) and morphological comparisons of the fungal isolates, the sequences of sample 1 showed maximum percentage of similarity with the sequences of the species *Aspergillus niger*, while sequence of sample 2 and 3 had higher percentage of similarity to the sequences of *Aspergillus flavus* and *Aspergillus oryzae* respectively **(Fig. 1a,b,c).**

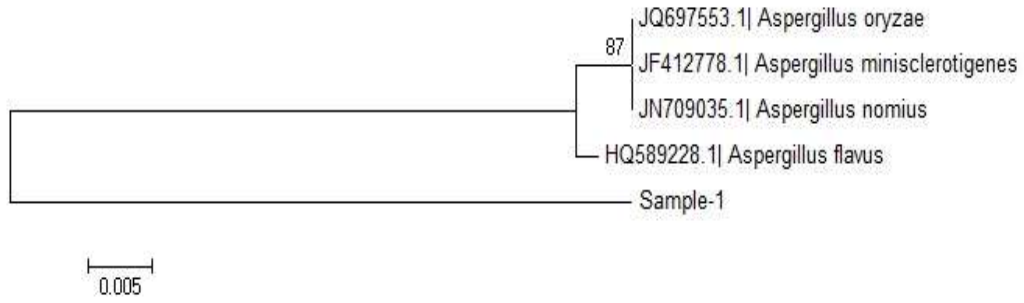

Fig. 1(a) Phylogenetic analysis of sample 1

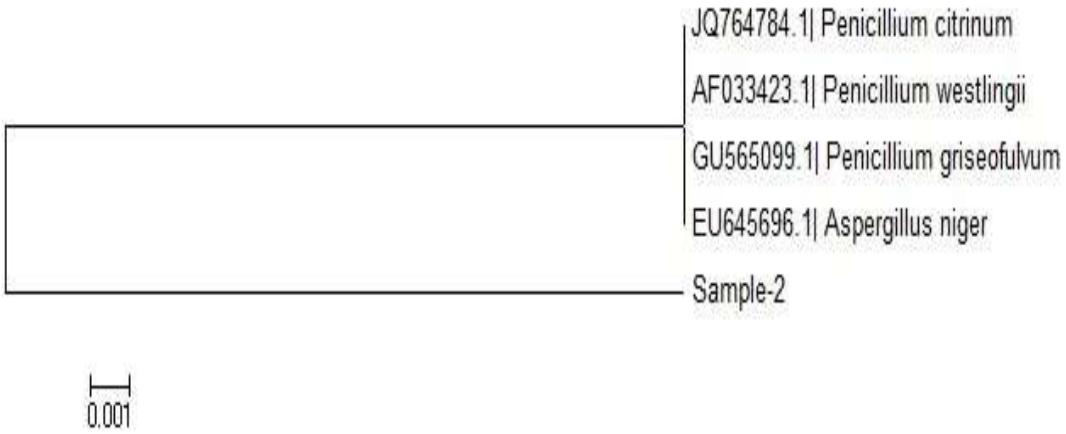

Fig. 1(b) Phylogenetic analysis of sample 2

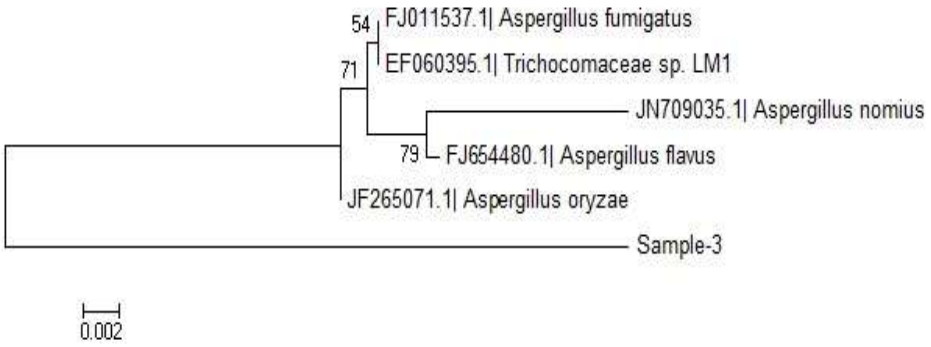

Fig. 1(c) Phylogenetic analysis of sample 3

Inhibition of fungi by Essential oils

In the agar dilution method (**Table 1)** all 10 essential oils exhibited fungi static effect on the test fungi. Vegetable oil used at 10-500 mg ml^{-1} as a control showed no inhibition on the test fungi. With an MIC of 0.02 µl ml^{-1} mustard oil was the most potent inhibitor. Eucalyptus oil and lemon grass oil had the least MIC of 50 µl ml^{-1} and 30 µl ml^{-1} respectively. Cinnamon oil, clove oil and lemon oil had MIC of 2 µl ml^{-1}, 4 µl ml^{-1} and 8 µl ml^{-1} respectively. Mustard oil, eucalyptus oil, lemon grass oil, cinnamon oil, clove oil and lemon oil had MICs similar in all the three test fungi. Citronella oil, camphor oil and peppermint oil had MIC of 6 µl ml^{-1} for *Aspergillus flavus* and rose oil had MIC of 8 µl ml^{-1}. Rose oil and peppermint oil had similar MIC for *Aspergillus oryzae* (8 µl ml^{-1}), citronella oil and camphor oil had 2 µl ml^{-1} and 12 µl ml^{-1} MIC respectively. MIC against *Aspergillus niger* was 12 µl ml^{-1} , 10 µl ml^{-1} , 4 µl ml^{-1} and 8 µl ml^{-1} for citronella oil, camphor oil, rose oil and peppermint oil respectively. Strong antifungal activity of these oils has been reported by many authors (Velluti *et al*.,2003 ; Wang *et al.*, 2005 ; Tzortzakis, 2009)

Table 1: Inhibition of fungi by essential oils

S.No.	Oil Name	Minimum inhibitory concentration (MIC)mm		
		Aspergillus niger **(µl ml^{-1})**	***Aspergillus oryzae*** **(µl ml^{-1})**	***Aspergillus flavus*** **(µl ml^{-1})**
1	Mustard oil	0.02	0.02	0.02
2	Cinnamon oil	2	2	2
3	Clove oil	4	4	4
4	Citronella oil	6	2	12
5	Camphor oil	6	12	10
6	Lemon oil	8	8	8
7	Lemongrass oil	30	30	30
8	Rose oil	8	8	4
9	Peppermint oil	6	8	8
10	Eucalyptus oil	50	50	50
11	Vegetable oil	No inhibition	No inhibition	No inhibition

Inhibition of molds by fungicides

Copper sulphate inhibited all three fungi with an MIC of 600 µg ml^{-1} on PDA. Orthophosphoric acid was also a strong inhibitor with an MIC of 2 µl ml^{-1},12 µl $ml^{-1}$and 14 µl ml^{-1} against *Aspergillus niger, Aspergillus oryzae* and *Aspergillus flavus* respectively. But biphenyl was a weak fungicide, when compared with other fungicides with an MIC of 260 mg ml^{-1} against all three fungi **(Table 2).**

Table 2: Inhibition of fungi by fungicides

S.No.	Name of fungi	Fungicides (Minimal Inhibitory Concentration)		
		Copper sulphate ($\mu g\ ml^{-1}$)	Orthophosphoric acid ($\mu l\ ml^{-1}$)	Biphenyl ($mg\ ml^{-1}$)
1	*Aspergillus niger*	600	2	260
2	*Aspergillus oryzae*	600	12	260
3	*Aspergillus flavus*	600	14	260

Zone of Inhibition of leaf sheath dipped in Essential oils

Even though all essential oils used exhibited zone of inhibition, relatively large inhibition was found in mustard oil **(Table 3).** It is observed that mustard oil was the significantly strongest inhibitor compared to all other essential oils. Cinnamon oil had the next highest zone of inhibition in all the three fungi. Clove oil also acted as a potent inhibitor. *Aspergillus oryzae* was more sensitive to mustard oil, lemon oil, clove oil, lemongrass oil and cinnamon oil. *Aspergillus niger* and *Aspergillus flavus* was highly sensitive to mustard oil and cinnamon oil. *Aspergillus flavus* was sensitive to lemon oil too. Peppermint oil showed medium inhibition in *Aspergillus oryzae* and was a weak inhibitor in *Aspergillus niger* and *Aspergillus flavus*. Rose oil reported as a medium inhibitor in all the three fungi. *Aspergillus oryzae* was highly sensitive to lemongrass oil while *Aspergillus flavus* and *Aspergillus niger* were moderately sensitive.

All the three fungi were resistant to citronella oil. *Aspergillus niger* and *Aspergillus flavus* were resistant to eucalyptus oil. In the case of camphor oil inhibitory effect was observed on *Aspergillus oryzae* and *Aspergillus flavus* when *Palmyra palm* leaf was employed while the same oil inhibited the growth of all the three fungi while *Areca palm* leaf sheath was used. *Aspergillus niger* was resistant to camphor oil, citronella oil and eucalyptus oil. Citronella oil had no inhibitory effect except *Aspergillus flavus* (*Areca palm* leaf sheath) in which it showed a very mild activity. While the effect of eucalyptus oil was observed *Aspergillus oryzae* and *Aspergillus flavus* were sensitive when *Areca palm* leaf sheath was employed while the same oil inhibited the growth of all the three fungi while *Palmyra palm* leaf was used. The antimicrobial agents in essential oils may either pass into the agar medium by diffusion or be released through evaporation in the headspace within the plate. This complex mechanism warrant further investigation.

Table 3: Zone of inhibition of leaf sheath dipped in essential oils

S.No.	Oil Name	Name of the Organism (Zone of inhibition in mm)					
		Aspergillus oryzae		*Aspergillus flavus*		*Aspergillus niger*	
		Areca palm	*Palmyra palm*	*Areca palm*	*Palmyra palm*	*Areca palm*	*Palmyra palm*
1	Mustard oil	28.66±8.08	18.66±3	33.66±2.08	24±1	17.33±8.73	19±2
2	Cinnamon oil	23.33±6.11	12±1.51	19±2	10.33±3.51	11±1	20±8.66
3	Clove oil	7.33±0.57	7.33±0.57	7.33±6.42	6±5.5	8±1.73	5±4.35
4	Camphor oil	-	7.33±6.42	-	7.66±6.80	-	-
5	Citronella oil	-	-	7.33±6.42	-	-	-
6	Peppermint oil	7.33±6.42	7.33±6.42	7.33±6.42	-	7.33±6.42	7.33±6.42
7	Lemon oil	26.33±3.5	18.33±1.5	-	-	14±3.60	10±2.64

		1	2				
8	Lemongrass oil	14.66±4.72	13±1.73	-	7.33±6.42	7.33±6.42	7.33±6.42
9	Rose oil	-	9.33±2.30	7.33±6.42	-	14±3.60	7.33±6.42
10	Eucalyptus oil	10±2.64	-	4±6.92	-	-	-

Zone of inhibition of fungicides against fungi

Aspergillus niger Aspergillus oryzae and *Aspergillus flavus* were resistance to biphenyl. CuSO4 and Orthophosphoric acid were potential fungicides. *Aspergillus niger* was sensitive to $CuSO_4$ and Orthophosphoric acid with a zone of measurement 15.33±0.57 mm and 10±1 mm respectively. In the case of *Aspergillus oryzae,* the zone of inhibition was 15.33 ± 0.57 mm and 34.66 ± 8.33 mm in $CuSO_4$ and OPA respectively. Whereas, *Aspergillus flavus* was moderately sensitive with a zone of 23.66 mm to $CuSO_4$ and 15.33 ± 0.57 mm to Orthophosphoric acid **(Table 4).**

Table 4: Zone of inhibition of leaf sheath dipped in fungicides

S. No.	Name of fungi	Fungicides (Zone of inhibition in mm)		
		Copper sulphate ($CuSO_4$)	Orthophosphoric acid	Biphenyl
1	*Aspergillus niger*	15.33±0.57	10±1	No inhibition
2	*Aspergillus oryzae*	15.33±0.57	34.66±8.33	No inhibition
3	*Aspergillus flavus*	23.66±1.52	15.33±0.57	No inhibition

Fungi growth on the leaf sheath

Essential-oil-coating on the surface is required for effective prevention of fungi on the surface of the *Areca palm* leaf sheath and *Palmyra palm* leaf, and so dip-treatment was employed for fungal testing (Matan, 2008). Growth of fungi on leaf sheaths dip-treated with mustard oil and vegetable oil was examined after incubation for 45 days. The average rating for mustard oil showed no growth, while the vegetable oil (control) showed growth in the three fungi within few days. Thus the essential oil showed fungi resistance for up to 45 days.

It should be noted that although oil treatment might have some effects on exclusion of moisture from the test specimens, fungal spores were able to germinate and grow on the control specimens dip-treated with vegetable oil. As a consequence, mechanisms other than moisture exclusion that are caused by components in essential oils should play a key role in inhibition of fungal spore germination.

Zone of Inhibition using vapour phase of Essential oil

The results tabulated in Table 5 reveals the growth of fungi on the PDA plates showed a zone of inhibition (ZI) at 25° C for 3 days. More effective results were observed against *Aspergillus oryzae* in mustard oil, clove oil, peppermint oil and lemon oil. Rose oil had no effect on *Aspergillus oryzae*. Peppermint oil and lemon oil had very low effect on *Aspergillus niger*, while the mustard oil had the highest effect. Mustard oil and peppermint oil were very effective against *Aspergillus flavus* than other oils. Vapour phase of mustard oil was efficient against all the three fungal agents. Vegetable oil (control) showed no inhibition. The use of essential oils in vapour phase was reported earlier by Amit and Anushree, 2010.

Table 5: Zone of inhibition on fungi growth using vapour exposure of essential oil

S. No.	Name of the oil	Name of the Organism (Zone of inhibition in mm)		
		Aspergillus oryzae	*Aspergillus niger*	*Aspergillus flavus*

1	Mustard oil	67.6±2.51	65.6±2.2	67.6±2.51
2	Cinnamon oil	42.33±2.08	33.66±1.52	31.33±1.53
3	Clove oil	67.6±2.51	31.33±1.53	24.33±1.53
4	Citronella oil	44±1	42±1	45±1
5	Camphor oil	43±1	42±1.53	31.7±1.53
6	Lemon oil	67.6±2.51	7.66±2.08	7.66±2.08
7	Lemongrass oil	7.66±2.08	34.66±1.15	12.33±2.08
8	Rose oil	No inhibition	18±1	12.33±2.08
9	Peppermint oil	67.6±2.51	6.33±0.58	67.6±2.51
10	Eucalyptus oil	21.66±3.06	15±1	12.33±2.08
11	Vegetable oil	No inhibition	No inhibition	No inhibition

IV. CONCLUSION

Fungi such as *Aspergillus niger, Aspergillus flavus* and *Aspergillus oryzae* isolated from diseased *Areca palm* leaf sheaths and *Palmyra palm* leaf were inhibited by Clove oil, cinnamon oil, mustard oil, peppermint oil, eucalyptus oil, citronella oil, camphor oil, rose oil, lemon oil and lemon grass oil. They were the fungal agents mainly responsible for the deterioration of the leaf products and spoilage of food packed in them. These extracted essential oils at their MICs were capable of inhibiting spore germination and growth of these fungi for at least 45 days in storage at 25 ˚C and 100% RH. So in future for use in food packaging and other handicraft products bio-based material such as *Areca palm* leaf sheath and *Palmyra palm* leaf treated with essential oils can be considered. Among the ten essential oils employed for screening, mustard oil was showed more potential than others. However, consumer sensory tests will be needed to determine concentrations of essential oils suitable for specific products.

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