Anti-Fungal Activities and Responses of Plant Essential Oils against Post-Harvest Disease of Mango (*Mangifera indica* L.) Fruit

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Abstract: *Post-harvest diseases of mango reduce fruit quality and cause severe yield losses with completely unmarketable fruits. A lab study was carried out to test the possibility of the use of some plant essential oils i.e. Artemisia indica Willd. Eucalyptus citriodora Hook., Cymbopogon citratus (DC) Stapff., Cinnamomum tamala (Buch-Ham.) Nees., and Zanthoxylum armatum DC. to reduce post-harvest losses induced by Colletotrichum gloeosporioides, Alternaria alternate, and Aspergillus niger. The pathogens were isolated from infected mango fruits collected from local markets. Pathogenicity test was confirmed by inoculating the pathogen into healthy mango fruit. The essential oils were extracted by hydro-distillation process using Clevenger oil extracting apparatus and were assayed under in-vitro condition by using various concentrations (2.5, 5, 10 and 40µl/ml)) against test fungi with controlled set to determine percent inhibition of mycelial growth of test fungi by poisoned food techniques. All five essential oils showed significant anti-fungal effect in reducing the fungal linear growth ($p<0.05$) at all tested concentrations over all tested fungi. Among them, Cinnamomum tamala showed the best performance of anti-fungal effect in controlling Colletotrichum gloeosporioides, Alternaria alternata and Aspergillus niger, which inhibited the mycelial growth by 95.45%., 89.82% and 90.57%, respectively at 40µl/ml concentrations.*

Keywords: *Anti-fungal activities and responses, Mango fruits, Plant essential oils, Post-harvest diseases*

Introduction

Supply of vitamins and minerals from fruits play a vital role in the human diet. Mango (*Mangifera indica* L.) is an important and widely cultivated sub-tropical and tropical fruit, native to India and South-east Asia (Mukherjee, 1953). It is known as "the king of fruits" because it is the most popular fruit in tropical regions due to its attractive color, texture and juicy flavor (Mehta, 2017). Post-harvest disease can reduce fruit quantity as well as quality because they yield completely unmarketable product and in many cases blemished fruits does not meet cosmetic standard for first quality fruit in major import market. Globally, post-harvest loss for cereal crop is only 30%, and 40-50 % of perishable vegetable and fruit crops (Food, 2015). In context of Nepal, post-harvest loss in food is 15-20% and 20- 40% for fruits and vegetables (PHMD, 2015).

Anthracnose is one of the most prevalent and severe disease of mango in most areas where the raised crops have been caused by *Colletotrichum gloeosperoides*. It mostly occurs in humid production areas (Ploetz & Freeman, 2009). *Alternaria* rot is another major cause of post-harvest loss, mainly occurs in arid environment (Johnson, 2008). *Aspergillus* rot, the next disease in mango affects the fruit after harvest, occurs due to mismanagement and carelessness during storage (Johnsons, 2008).

Essential oils are a rich source of bio-active compounds with antifungal effects for both fungi against both pathogens and spoilage fungi (Daferera et al., 2003; Piccaglia et al., 1993). These oils are also bio-degradable and non-toxic (Adebayo et al., 2013). Naturally occurring biologically active compounds from plants are generally assumed to be more acceptable and less hazardous than synthetic compounds and represent a rich source of potential disease control agents (Tripathi et al., 2008). (Lee et al., 2008) tested 39 essentials for antifungal activities as volatile compounds against five phyto-pathogenic fungi at a dose of 1 µl plate^{-1}. This study aimed to evaluate the effectiveness of a number of plant essential oils as alternative method to synthetic pesticides against growth of test fungus, the causal agent for post-harvest disease for mango fruit under *in-vitro* conditions.

Materials and Methods

Source of Tested Plant Essential Oils

Leaves of *Eucalyptus citriodora* (Masala), *Cinnamomum tamala* (Tejpat), *Artemisia indica* (Titepati) were collected from TU, Kirtipur, Kathmandu, *Cymbopogon citratus* (Lemon grass) was collected from Baniyataar, Kathmandu and *Zanthoxylum armatum* (Timur) from Champadevi, Kathmandu. The collected foliar parts of the plants were dried in shade for 24 hours and then leaves were surface sterilized. About 100 g of leaf sample was then grounded to make pulverized and subjected to hydro-distillation for 6-8 hours in Clevenger's apparatus in 1000 ml water. Two layers:

upper aromatic layer of essential oil and lower colorless, were observed and the aromatic layer was collected and dehydrated over anhydrous sodium sulphate and stored at <10˚C temperature as described by (Rao & Srivastava, 1994). The stock solution of 100µl of essential oils was diluted with 60% Acetone to make five different concentrations of 40µl/ml, 20µl/ml, 10µl/ml, 5µl/ml and 2.5µl/ml. The following method was implemented to make the diluted volume using formula;

$$V_1S_1 = V_2S_2$$

Where, V_1= Volume of stock solution

S_1= Concentration of stock solution

V_2=Volume of final solution

S_2= Concentration of final solution

Isolation and Identification of test fungi (*Colletotrichum gloeosporioides*, *Alternaria alternata* and *Aspergillus niger*)

Some pieces of fungal colony from infected mango (Figure 1, 2 and 3), collected from local market were transferred aseptically on Potato Dextrose Agar (PDA) media. After one week, the observed colonies were identified (Figure 4, 5 and 6) by using standard literatures (Barnett & Hunter, 1972; Ellis, 1971; Watanabe, 2010).

Fig 1: Mango infected with *Colletotrichum gloeosperoides*

Fig 2: Mango infected with *Alternaria alternata*

Fig 3: Mango infected with *Aspergillus niger*

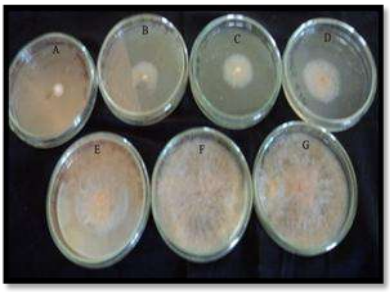


Fig 4: Antifungal activity of *Cymbopogon citratus* against *Colletotrichum gloeosperoides*

Fig 5: Antifungal activity of *Zanthoxylum armatum* against *Alternaria alternata*

Fig 6: Antifungal activity of *Cinnamomum tamala* against *Aspergillus niger*

Pathogen Culture

Initially, the diseased fruits were cleaned with sterile water. After air-drying, the fruit was sterilized by 70% ethyl alcohol with the help of cotton and with distilled water and then allowed to dry. The small pieces from affected portion (only epicarp) of each sample was taken with the help of sterilized forceps and plated under aseptic condition on PDA. The plates were incubated at 25± 2ºC for one week. After one week, the mycelial growth of fungal colony was observed. The pure culture of most frequent pathogen *Colletotrichum gloeosporioides*, *Alternaria alternata* and *Aspergillus niger* were preserved by sub-culturing in PDA media and incubated at 25±2ºC. Similarly, fungi were inoculated in agar slant and stored at <10ºC temperature for the preservation of their vigor and long-term storability/ applicability.

Fungal toxicity

The fungal toxicity of the essential oils was assessed by poisoned food technique (Grover and Moore, 1962). It is the process of testing the antifungal effect by poisoning the media with oil and extract. Each concentration of 0.5 ml essential oil was aseptically poured into sterilized petriplates followed by the addition of 9.5ml of PDA. The petriplates were swirled gently to allow proper mixing of the contents. After the solidification of the media, each petriplate was then inoculated aseptically upside down at the center by 4 mm diameter of tested fungus. Positive and Negative control were maintained in this regard. In positive control set, 60% Acetone was used, whereas in

Negative control, no essential oil was used. The inoculated petriplates were incubated for one week at 25±2ºC. The average diameter was measured by using measuring scale on the 7th day of incubation and the percentage of mycelial growth inhibition was calculated by the following formula:

$$\text{Percentage Inhibition} = (Gc - Gt) / Gc \times 100\%$$

Where, Gc = growth of mycelial colony after incubation in control set (Diameter of colony in control set-diameter of Inoculum disc).

Gt = growth of mycelial colony after incubation period in treatment set (Diameter of colony in treatment set-diameter of Inoculum disc).

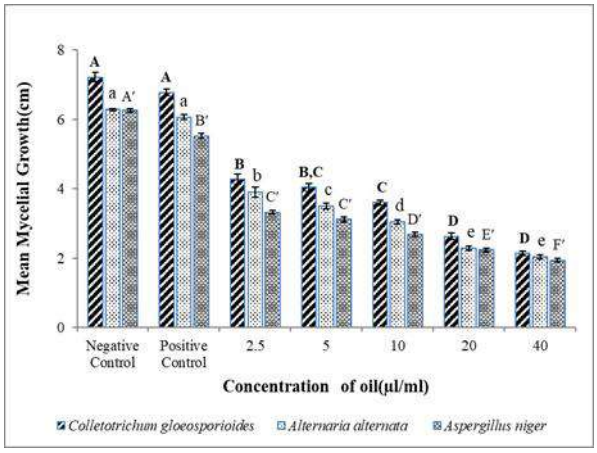
Statistical Analysis

Data entry, chart diagrams and graphs were accomplished by using Microsoft Excel, 2007. The data were analyzed using ANOVA test by using SPSS v.20 at confidence level 95%, i. e. at 5% significance level. Altogether, 450 sample tests were carried out for mycelial growth control measurement by essential oils at all concentrations on fungi tested with 5 times replication of each essential oil at particular concentration to each fungus. To compare the differences, Post-Hoc; Bonferroni test was done.

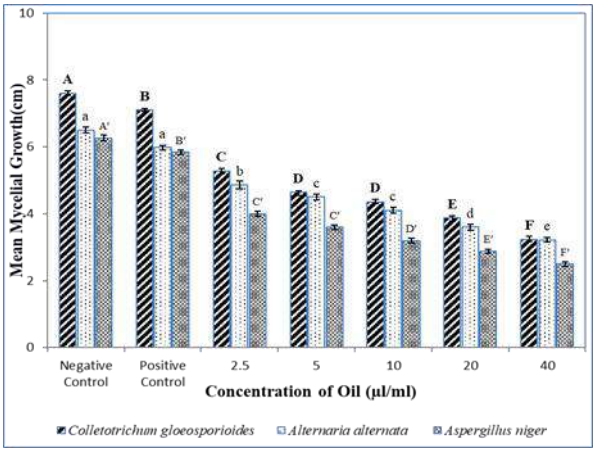
Results

Mycelial growth of test fungi under different plant essential oils

The result showed that all five plant essential oils had significant antifungal effect ($p<0.05$) on mycelial growth of all three test fungi (Figure 7 (A-E). Among them, oil of *Artemisia indica* and *Eucalyptus citriodora* were the most effective antifungal activity on *Aspergillus niger* followed by *Alternaria alternata* and *Colletotrichum gloeosporioides* (Figure 7 (A) and (B). Meanwhile, oil of *Cinnamomum tamala* was most effective on *Colletotrichum gloeosporioides* (Figure 7 (C). Oil of *Cymbopogon citratus* was more effective to control the Mycelial growth caused by *Colletotrichum gloeosporioides* (Figure 7 (D). Finally, oil of *Zanthoxylum armatum* was significantly effective to control the Mycelial growth due to *Alternaria alternata* (Figure 7 (E). The positive control (Acetone) was comparatively very less effective for all three fungi than all five essential oils at 2.5µl/ml concentration. Statistically, two-way ANOVA (Table 1) showed that different types of plants' essential oil and their different concentrations were effective significantly to control mycelial growth caused by *Colletotrichum gloeosporioides, Alternaria alternata* and *Aspergillus niger*.



(A)



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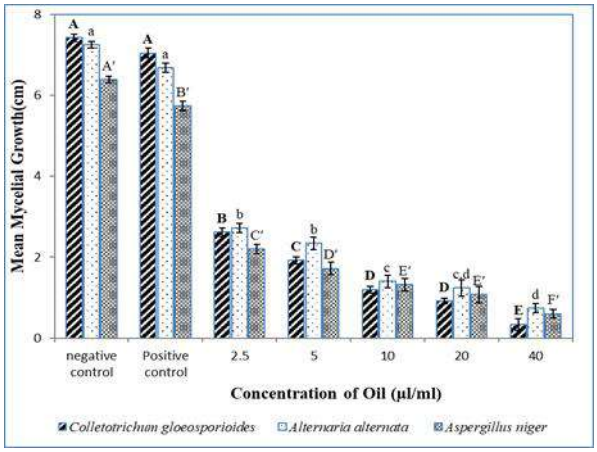
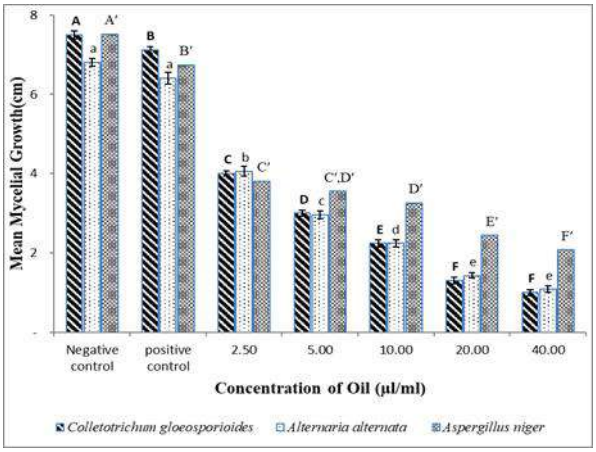
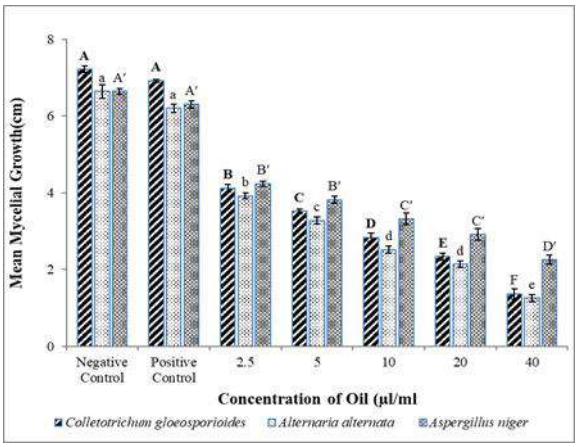


(C)



(D)



(E)

Fig 7: Antifungal activity of *(A) Artemisia indica (B) Eucalyptus citriodora (C) Cinnamomum tamala (D) Cymbopogon citratus (E) Zanthoxylum armatum* against *Colletotrichum gloeosporioides, Alternaria alternata* and *Aspergillus niger*.. The mean values sharing same alphabet (bold capital letter for *Colletotrichum gloeosporioides*, small letter for *Alternaria alternata* and capital letter with prime sign for *Aspergillus niger* are not significantly different from each other according to Bonferroni test at (P< 0.05). Error bar represents the standard error of means.

Percentage Inhibition of Test Fungi by different Essential Oils

Among all tested concentrations of all plant essential oils, Cinnamomum *tamala* oil was significantly reducing the fungal linear growth of *Colletotrichum gloeosporioides* (Figure 8). In case of 2.5µl/ml concentration, the oil of *Cinnamomum tamala* showed the highest inhibition (64.69%) which was followed by *Cymbopogon citratus* (53.9%) and descending orders performance were *Zanthoxylum armatum* (42.67%), *Artemisia indica* (40.50%) and *Eucalyptus citriodora* (30.53%), respectively. In case of 5µl/ml concentration, the oil of *Cinnamomum tamala* oil showed the highest inhibition (74.13%) which was followed by *Cymbopogon citratus* (59.67%), *Zanthoxylum armatum* (51.22%), *Artemisia indica* (43.99%) and *Eucalyptus citriodora* (38.91%), respectively. Similarly, at 10µl/ml concentration, the performance was similar like oil of *Cinnamomum tamala* has the highest inhibition (83.06%) followed by Cymbopogon citratus (70.15%), *Zanthoxylum armatum* (60.69%), *Artemisia indica* (50.05%) *and Eucalyptus citriodora* (42.6%), respectively. Meanwhile, at 20µl/ml concentration oil of *Cinnamomum tamala* showed the highest inhibition (87.6%) followed by Cymbopogon citratus (82.64%), *Zanthoxylum armatum* (67.54%), *Artemisia indica* (63.60%) and *Eucalyptus citriodora* (48.95%), respectively. Again, at 40µl/ml, *Cinnamomum tamala* showed the highest inhibition (95.45%) followed by *Cymbopogon citratus* (86.64%), *Zanthoxylum armatum* (81.04%), *Artemisia indica* (70.31%) and *Eucalyptus citriodora* (57.36%), respectively.

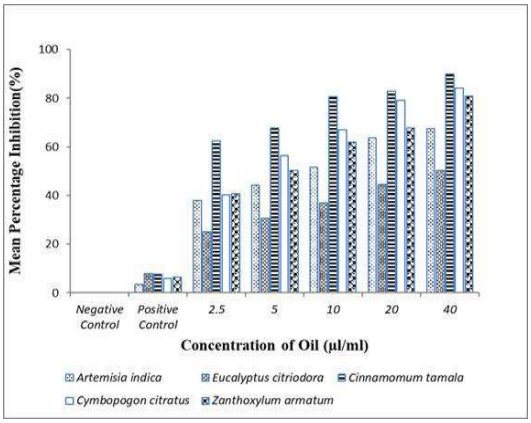


Figure 8: Fungal toxicities against *Colletotrichum gloeosporioides*

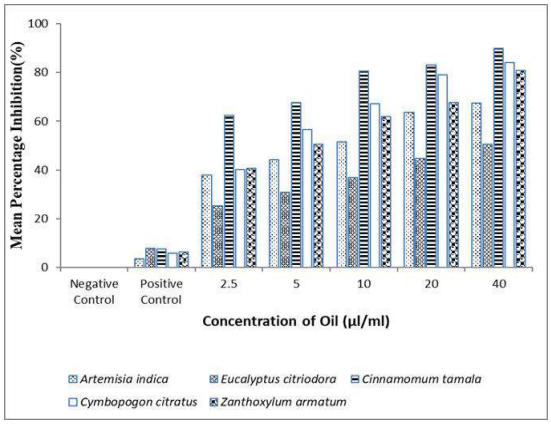


Figure 9: Fungal toxicities against *Alternaria alternate*

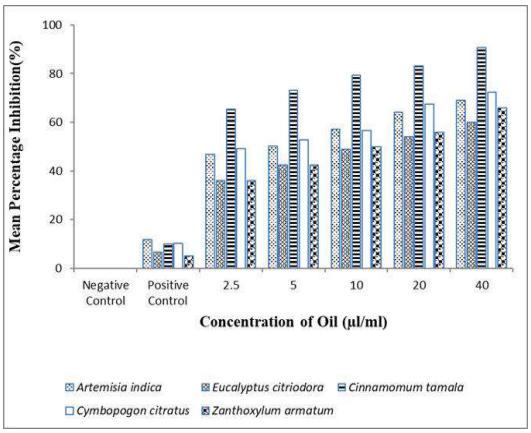


Figure 10: Fungal toxicities against *Aspergillus niger*

The oil of *Cinnamomum tamala* showed the best antifungal activity to control *Alternaria alternate* (Figure 9). At 2.5µl/ml concentration, *Cinnamomum tamala* showed the highest inhibition (62.46%) which was followed by *Zanthoxylum armatum* (40.75%), *Cymbopogon citratus* (40.16%), *Artemisia indica* (37.87%) and *Eucalyptus citriodora* (25.22%) respectively. Similarly, at 5µl/ml concentration, oil of *Cinnamomum tamala* showed the highest inhibition (67.74%) that was followed by *Cymbopogon citratus* (56.47%), *Zanthoxylum armatum* (50.43%), *Artemisia indica* (44.28%) and *Eucalyptus citriodora* (30.74%) respectively. At 10µl/ml concentration also oil of *Cinnamomum tamala* showed the highest inhibition (80.62%) that was followed by *Cymbopogon citratus* (67.08%), *Zanthoxylum armatum* (61.84%), *Artemisia indica* (51.61%) and *Eucalyptus citriodora* (36.90%) respectively. Meanwhile, at 20µl/ml concentration also the oil of *Cinnamomum tamala* showed the highest inhibition (82.96%) which followed by *Cymbopogon citratus* (79.05%), *Zanthoxylum armatum* (67.69%), *Artemisia indica* (63.70%) and *Eucalyptus citriodora* (44.61%) respectively. Again, at 40µl/ml concentration also oil of *Cinnamomum tamala* showed the highest inhibition (89.82%) followed by *Cymbopogon citratus* (84.12%), *Zanthoxylum armatum* (80.91%), *Artemisia indica* (67.51%) and *Eucalyptus citriodora* (50.42%) respectively. (Figure 9).

Comparatively, *Cinnamomum tamala* was more toxic to *Aspergillus niger* from lower (2.5µl/ml) to higher concentration (40µl/ml) (Figure 10). At 2.5µl/ml concentration, *Cinnamomum tamala* showed the highest inhibition (65.45%) which was followed by *Cymbopogon citratus* (49.25%), *Artemisia indica* (46.95%) *Zanthoxylum armatum* (36.08%) and *Eucalyptus citriodora* (36.01%), respectively. Similarly, at 5µl/ml concentration, oil of *Cinnamomum tamala* showed the highest inhibition (73.07%) that was followed by *Cymbopogon citratus* (52.70%), *Artemisia indica* (50.13%), *Eucalyptus citriodora* (42.47%) and *Zanthoxylum armatum* (42.44%), respectively. At 10µl/ml concentration also oil of *Cinnamomum tamala* showed the highest inhibition (79.34%) that was followed by *Artemisia indica* (57.18%), *Cymbopogon citratus* (56.59%), *Zanthoxylum armatum* (49.98%) and *Eucalyptus citriodora* (48.90%), respectively. Meanwhile, at 20µl/ml concentration, the oil of *Cinnamomum tamala* showed the highest inhibition (83.09%) which followed by *Cymbopogon citratus* (67.51%), *Artemisia indica* (64.19%), *Zanthoxylum armatum* (55.97%) and *Eucalyptus citriodora* (53.99%), respectively. Again, at 40µl/ml concentration also oil of *Cinnamomum tamala* showed the highest inhibition (90.57%) followed by *Cymbopogon citratus* (72.29%), *Artemisia indica* (68.98%), *Zanthoxylum armatum* (60.03%) and *Eucalyptus citriodora* (57.66%), respectively (Figure 10).

Antifungal Activity Test

Table 1. Two way ANOVA test for the analysis of anti-fungal activities and their responses

Descriptions	Test Applied	Degree of freedom	p-value of *Colletotrichum gloeosperoides*	p-value of *Alternaria alternate*	p-value of *Aspergillus niger*
Effect of different types of Oil	Two Way ANOVA	4	<0.001	<0.001	<0.001
Different concentration of Essential Oils		6	<0.001	<0.001	<0.001

Two-way ANOVA (Table 1) showed that there was significant difference on different types of oil and different concentration on treatment of *Colletotrichum gloeosperoides, Alternaria alternate and Aspergillus niger* at 0.05 level of significance and could be well described that various plant essential oils and their graded concentration levels are significantly effective on controlling the anti-fungal activities and giving the positive significance in controlling the various fungal diseases caused by various fungus.

Discussions

The most active oil for reducing the growth of all the tested fungi with significant value under *in-vitro* condition was *Cinnamomum tamala* oil, followed by *Cymbopogon citratus* oil at all concentrations. *Eucalyptus citriodora* oil showed a lower effect at all tested concentrations to control all tested fungi compared to other plant essential oils. However, it showed the positive result in inhibiting the growth of tested fungi than control treatment. Phenyl-propanoids have been reported to be the major component (about 66%) in *C. tamala* leaf oil, which include eugenol, cinnamaldehyde, cinnamyl alcohol, cinnamyl acetate and cinnamic acid. In addition, several other components such as B-caryophyllene, spathulinol, sesquiterpenoids and viridiflorene etc. are also present in appreciable quantity in the oil (Chanotiya and Yadav 2010; Kapoor et al. 2009). Presence of these chemical moieties in *C. tamala* leaf oil may also be accounted for higher anti-fungal properties than other essential oils. Natural phytochemicals present in *C. tamala* leaf extracts have potential to prevent growth of food spoilage/ pathogenic fungi. Lemongrass oil (ranging between 25 and 500 ppm) shows strong anti-fungal against five fungus including *Aspergillus niger* (Tzortzakis and Economakis, 2007). Mycelial growth of *Aspergillus niger* was completely inhibited using 1.5 µl/ml or 2.0 µl/ml of *Cymbopogon citratus* essential oil. Satyal et al. (2012) also found marginally antifungal against *Aspergillus niger* (MIC=313 µg/ml) and suggested the anti-fungal activity of *A. indica* oil may be due to high concentration of ascaridole (15.4%), isoascaridole (9.9%), trans-p-mentha-20, 8-dien-1-ol (9.7%) and trans-verbenol (8.4%). Major components of *Zanthoxylum armatum* are linalool (56.10%) and methyl cinnamate (19.73%) because of their fungi static nature, could be useful in inhibition of growth of fungi (Prakash et al. 2012). The anti-fungal activity may be due citronella (49.45%) and citronellal (11.86%) as main components from the leaf essential oil of *Eucalyptus citriodora* (Su et al. 2006).

Conclusions

Plant essential oils used in the study showed possibility to reduce post-harvest loss in mango fruits by inhibiting the mycelial growth of fungi. The most active oil for reducing the growth of all the tested fungi with significant value under vitro condition was *Cinnamomum tamala* oil followed by *Cymbopogon citratus* oil at all concentrations. *Eucalyptus citriodora* oil showed a lower effect at all tested concentrations to control all tested fungi compared to other oils. However, it showed positive result in inhibiting the growth of tested fungi than control treatment. Essentail Oils, which are potential source of sustainable eco-friendly botanical fungicides, may attract the attention of scientific community and food industries in place of synthetic preservatives for possible application in the enhancement of shelf life of various food items by protecting them from molds degradation during long-term storage.

Acknowledgements

The authors would like to thank Prof. Mohan Siwakoti, HoD, and Central Department of Botany TU for availability of laboratory facilities. We are grateful to Dr. S. K. Jha, Dr. C. P. Pokhrel and Dr. L. B. Thapa, for their valuable feedback and suggestions.

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