Shelf life and efficacy of antifungal plant based extract and formulations

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***Abstract*— Commercial viability of any natural or plant based formulations depends on its shelf life. Storage for prolonged perid under different temperature ranges, pH and sunlight intensity may leads the changes in efficacy of secondary metabolites it contained which in turn altered the antimicrobial efficacy. Hence it is very important to check efficacy of plant based formulations under varying physical condition. In the present work, it was observed that active content of partially purified benzene extract and subsequently prepared formulations remain active under varying physical storage conditions. Storage did not affectthe efficacy as well which was confirmed via cytomorphology of treated fungi i.e. *Alternaria alternata*. Thus one more fact for successful application of plant based formulation is their physical stability under environmental condition.**

***Index Terms:* Commercial viability, physical condition, storage, formulation, cytomorphology.**

INTRODUCTION

Plants possess a variety of antimicrobial defense mechanisms. Antimicrobial activity of plants depends on the chemical nature of compounds present in them. In order to develop bioformulation, it is very important to determine that extrcaat remain stable under various physical condition. Extended shelf life of plant based formulations play very important role in commercial viability of the same. Extended viability confirmed when extract retained its antimicrobial property which can be proved by studying cytomorphology of pathogens against which proposed extract had been developed.

Infection by *A. alternata* causes the development of small, circular, necrotic spots on fruits and leaves of the plant which quickly convert into typical concentric rings (Kannaiyan and Nene, 1977). In later stages of infection blighting in leaves develop which results in defoliation and plant death (Harris *et al*., 2009; Tatiana *et al*., 2010).

The ability of plants to produce various types of secondary metabolites helps them to fight invading microorganisms and they work as main agents for plant defense mechanisms against disease-causing pathogens (Cowan, 1999). Various researchers elicit the antimicrobial activity of the plant secondary metabolites which is useful for the growth inhibition of invading pathogens (Reddy *et al.*, 2009; Reddy *et al.*, 2010; Dissanayake *et al*., 2013; Seasan *et al*., 2015; Perveen et al., 2020).

Importance of use of plant extracts in herbal formulations depends on the stability of physical and chemical properties. The extract should not undergo any drastic change due to change in temperature, pH or exposure to sunlight. It should have a long shelf life of at least six months and there should not be reduction in its antimicrobial activity during this period.

Several workers have studied the effect of physical factors on extract efficacy. Nishad (2022) studied the effect of physical factors like thermal, nonthermal, and physicochemical factors of temperature, pH, pressure, sonication, radiation, electric field, humidity, oxygen, and light on stability of plant extract. It was emphasized that no variability in efficacy of extract enhance its commercial viability. Hada and Sharma(2017) studied the effect of

sunlight, heat, pH and long-term storage on extract and herbal formulation. Ranganathan and Balajee (2000) investigated the effects of different physical factors on the biological activities of ethanolic extract of *Ocimum sanctum* and *Cassia alata*. The extract of black mulberry fruits *Morus nigra* possesses a high amount of antioxidant activity.

The authors also reported a reduction in antimicrobial activity at alkaline pH. Mehrotra *et al*., (2010) reported the stability of bioactive components present in the ethanolic extract of *Syzygium aromaticum* over a wide range of pH and temperature values.

In the present work, Moringa *oleifera* leaf extracts based formulation has been developed against *Alternaria alternata* causes disease in tomato. It was kept under various physical condition for extended period of time and checked for efficacy by studing cytomorphology of treated pathogen.

Materials and Methods

EFFECT OF PHYSICAL FACTORS ON THE ANTIFUNGAL ACTIVITY OF BIO FORMULATIONS

Effect of sunlight

To study the impact of sunlight on the viability of extracts and bio formulations, the exposed vials were tested for their antifungal activity using a two-fold serial dilution method and poison food technique (Wang and Ke-Qiang, 2001). The percent mycelial inhibition activity was measured according to equation 1. Efficacy was also checked by cyomorphology changes in test pathogen.

Mycelial growth inhibition (%) = [(Gc – Gt)/ Gc] × 100 Equation 1

Where Gc = Average diameter of the fungal colony after 7days of incubation in control plates after subtracting the diameter of inoculums disc.

Gt = Average diameter of the fungal colony after 7days of incubation in poisoned plates (plates in which bio formulation was added with media) after subtracting the diameter of inoculums disc.

Effect of heat

To study the effect of dry heat sterile glass vials containing 50% alcoholic crude extract, partially purified benzene extract and four different bio formulations were placed into a hot air oven with two different temperatures viz. 40°C and 90°C for 4h. Similarly, the effect of wet heat was assessed by placing the same set of vials into the water bath at 50 °C and 100 °C temperatures separately for 4h. The set of vials placed at room temperature (untreated) was served as control. Afterward using the poison food technique, the percent mycelial inhibition activity was evaluated according to equation 1 and data compared with the control.

Effect of pH

PDA media was added into the tubes containing extract and bio formulations (With different pH) and further inoculated with *Alternaria alternata*. After inoculation, the tubes were incubated at 28±1 °C temperature for 72h. After completion of incubation, the tubes were assayed for change in MIC (Minimum Inhibitory Concentration) of bio formulation extracts.

Effect of storage time

Extract and bio formulations were stored at room temperature maximum for 12 months. The change in their antifungal activity was measured at regular intervals of 6 months and maximum up to 12 months using the two-fold serial dilution and poison food technique. Percent mycelial growth inhibition activity of biomaterials was calculated using equation 1.

Efficacy was also checked by cyomorphology changes in test pathogen.

STATISTICAL ANALYSIS

All the experiments were performed in triplicates, repeated thrice via a randomized design. The obtained experimental data were statistically analyzed with IBM SPSS Statistics Ver. 20 software. The statistical data were expressed as the mean of three independent replications ± standard error (SE).

EFFECT OF EXTRACT ON THE MORPHOLOGY OF TEST FUNGI *ALTERNARIA ALTERNANTA*

The effect of partially purified benzene extract (kept for long time) of *M. oleifera* leaf powder on various morphological and cytological parameters of *Alternaria alternata* was studied. The test tubes containing an equal amount of fungal culture was inoculated with increasing concentration of from 10mg/ml to 0.019mg/ml until the MIC was reached. After incubation for 7 days at 28) ± 2^0c the aliquot of

the treated culture was stained with cotton blue and mounted in lactophenol. The small amount of fungal biomass containing mycelium and spores was harvested from each tube for microscopic examination. Morphological changed such as mycelium width, conidia size, and numbers of conidia were observed with an Olympus trinocular research microscope BX- 51 and an ocular micrometer. A hemocytometer was used to count the numbers of conidia and spores. The data were recorded and morphological changes were compared with the concentration changes of the extract which demonstrate the effect of benzene extract of *M. oleifera* on cytomorphological features of *A. alternata*.

RESULT AND DISCUSSION

The effect of different physical factors on the antifungal activity of *M. oleifera* leaf extracts and their screened bio formulations have been assessed by the food poison technique. In case of exposure to the sunlight, there were no significant changes in the activity of bio formulations after the sunlight exposure for 15 hrs and 30 hrs. Antifungal activity remained sustainable for benzene extract as well, but in the case of alcoholic crude extract the activity was reduced for the sample exposed in the sunlight for 30 hrs, percent of mycelial growth inhibition activity was reduced from unexposed to exposed in the sunlight for 30 hrs.

In case wet heat treatment (treated in a water bath) for 50 °C and 100 °C, except benzene extract all the other samples showed a reduction in their antifungal activity at high-temperature ranges. After heat exposure at higher temperature ranges (90 °C and 100 °C) antifungal activity of alcoholic crude extract and all the four bio formulations were decreased. These results are in agreement with the antimicrobial activity of the same plants subjected to different water stress regimes did not differ significantly (Netshiluvhi and Eloff, 2016). Mikayel (2017) suggested that thermostability of extract depends on presence of theromolabile constituents in extract which did not altered at temperature ranges and retained activity.

The effect of pH on the antifungal efficacy of the selected materials was evaluated. It was observed that when pH increased up to pH 9 and decreased up to pH 4 in both conditions the antifungal activity of the studied materials reduced when compared with the values of pH 7. Except benzene extract of *M. oleifera* leaves. On the contrary minimum loss of activity was found with formulation-19, showed a reduction in activity from71.35% to 71.28%. Lee *et al.* (2004) investigated heat and pH susceptibility of Chinese leaf extracts and found that heat treatment above 75°C reduced the inhibitory activity while inhibitory activity is stable between pH 2.0 to 8.0. Similarly Di Mambro *et. al.* (2005) studied the combined effect of temperature and relative humidity on the antioxidant effect of different plant extracts. Ali (2010) also described that antimicrobial activities of aqueous and methanolic extracts from *Salvia officinalis* and *Salix acmophylla* reamin afte storage at acidic pH.

Table 1 Effect of various physical factors

S.no	Extract	pH	Temperature (Wet and dry)	Sunlight	Storage time
1	50% Alcoholic Crude extract	RA	RA	RA	RA
2	Partially purified extract (PE)	UA	UA	UA	UA
3	Bio formulation No. 2	UA	UA	UA	UA
4	Bio formulation No. 13	UA	UA	UA	UA
5	Bio formulation No. 19	UA	UA	UA	UA
6	Bio formulation No. 24	UA	UA	UA	UA
7	Control(Without Extract)	UA	UA	UA	UA

RA= Reduced activity

UA= Unaltered activity

The effect of antifungal activity of leaf extracts of *M. oleifera* and their bio formulations was assessed against the storage time of biomaterials. All the biomaterials were stored at room temperature for 12 months and their antifungal activity was assessed in 6 months intervals. For all the studied biomaterials it was observed that the storage time did not affect their antifungal activity as no significant changes in the growth of fungal colonies and percent mycelial

inhibition activity was observed with increased storage time. Griggs et al (2010) described that extract prepared from some medicinal plant remain partially activity after six years of storage which similar to present study in which extract leads changes in cytomorphology of pathogen after 6 months of storage. Similar findings given by Al-Zahrani et al. (2016).

EFFICACY OF PARTIALLY PURIFIED BENZENE EXTRACT (STORED AT ROOM TEMPERATURE FOR 12 MONTHS) OF *M. OLEIFERA* LEAVES ON THE CYTOMORPHOLOGY OF *A. ALTERNATA* FUNGI

The experiment was conducted to evaluate the effect of partially purified benzene extract (stored for 12 months) of *M. oleifera* leaves on cytomorphological features of *A. alternata.* The data are presented in Figures 5.1. In the control set where distilled water was added with the culture of *A. alternata* a profuse mycelial growth in the test fungi was observed. Thick mycelial mat, circular, dark black-brown and cottony colony growth were observed. Microscopic examination showed the presence of dark, unbranched conidiophores which were broader than the vegetative hyphae. Conidiophores were bearing chains of conidia which were dark brown and exhibit transverse and longitudinal septa with distinct beak (Fig. 5.1 A). As the concentration of the extract was increased from 10mg/ml to 0.019mg/mla sequential decrease in the size of conidia and increase in the width of mycelia were observed. There were very few conidia left they have damaged conidia and their size and number were highly reduced (Fig. 5.1 A-C). Stephen et al (2013) also stated that minimum inhibitory concentration (MIC) values obtained against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the stored plant materials were generally either lower or roughly the same as in the fresh material. Thus storage for long period had not much effect on antimicrobial efficacy. Ranglová et al (2015) also observed that storing for particular time period did not affect the antimicrobial efficacy of plant. They worked on Extracts of T. Violacea for that.

It was observed that after 12 months of storage active extract i.e. partially purified benzene and formulations formulated of the same. Reduction in conidia number observed as the concentration of stored extract increased.

Fig. 5.1:MorphologicalAlterationsin *Alternariaalternata*DuetoTreatmentwithBenzeneExtractonDifferentConcentrations

A:Mycelium,ConidiaandConidiophoresof*Alternariaalternata*(Controlat400 x)

B: 1. NormalMycelium(at 400 x)
2. Myceliumshowingincreasedwidth(at400x)

C: 1. NormalConidia(at400x)
2. Conidiashowingdecreasedsize(at400x)

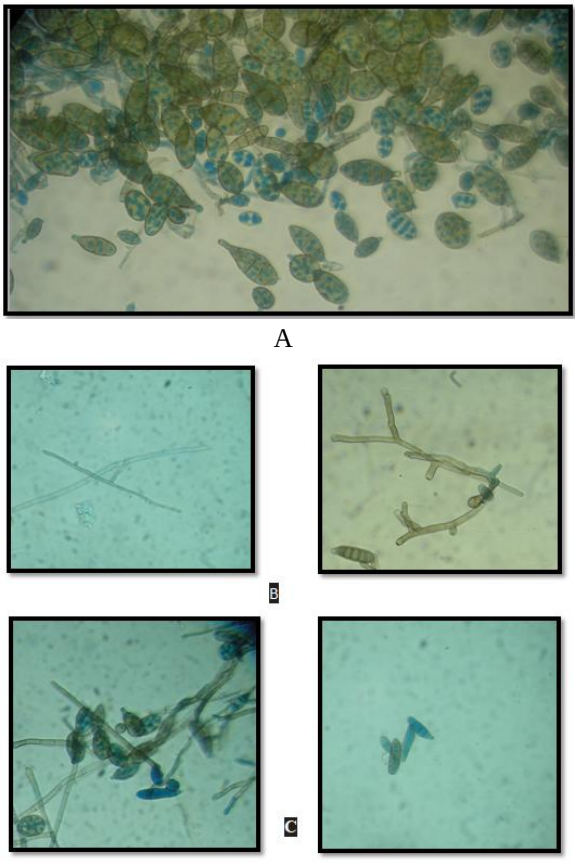

Fig. 5.1

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