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Research Article

Study on: The Effect of Various Physical Factors on the Stability of Natural Antifungal Agents

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Abstract

Variation in environmental factors is also played a major role in the quantification and qualitative measurement of active principles of the plants. The present study is carried to determine the effect of light, temperature, pH and storage condition on *Eucalyptus globulus* leaf extracts and bioformulation and its stability as the change in minimum inhibitory concentration of partially purified petroleum ether extract and activity zone of 100% alcoholic crude extract and bioformulation against the *Alternaria solani*. The activity of secondary metabolites is very responsive to change in the physical environment. Results showed that 15hrs exposure had no effect but after 30h exposure a slight reduction in the activity of 100% alcohol crude extract. There was no adverse effect of wet as well as dry heat up to 50°C of wet heat and 40°C of dry heat on extract and bioformulation efficacy however, heating at 100°C of wet heat and 90°C of dry heat for 4 hrs resulted in a slight reduction in the extract and bioformulation efficacy as a negligible growth of test fungus were observed. Reduction in antifungal activity of plant extract as well as bioformulation at acidic pH against *Alternaria solani* was observed. Storage for 6 and 12 months had no negative effect on extract and bioformulation efficacy and the antifungal activity was observed similar to freshly prepared extract activity. The present study concluded that the plant disease or plant pathogens can be controlled by plant extract and plant based bioformulations by increasing the shelf life with some little changes in the physical parameters such as light, temperature, pH and storage.

Keywords: *Environmental Factors; Bioformulation; Secondary Metabolites; Plant Pathogen; Antifungal Agents*

Introduction

Various physical factors affect the nature and activity of active phytochemicals present in the plant which are directly related to the plant's therapeutic potential [1]. The major alteration and changes in biochemistry and cytology of microbes are affected by secondary metabolites. During extract preparation, intense heating is applied, which can be changed the properties of active molecules of plant extract which might influence their respective activity [2,3].

If Bioformulation stability can be maintained at varying natural conditions then it can be truly economical and commercially viable. By the changes in temperature, pH or exposure to sunlight the chemical and physical properties of this bioformulation should not be changed

or it should have a long shelf life of at least six months and antifungal activity of this bioformulation also be remain as its original mode [4] work have been done regarding the stability of extract in the presence of different physical factors [5,6]. Reports are available regarding the effect of temperature on antimicrobial activity [7,8].

Hence it is very important to determine the effect of abiotic factors on extract as well as bioformulation, to improve their shelf life. Some workers have checked the stability of extract and bioformulation in a varying conditions of temperature and pH [9]. Stable activity of the combination of ethanolic extract of *Ocimum sanctum* and *Cassia alata* was investigated by researchers [10].

The literature survey revealed the effect of heat treatment pH and storage on the stability of plant extract activity [11]. Some research suggested that temperature ranges of 30°C to 100°C had no effect on the antimicrobial activity of the chloroform extract and activity slightly increased at acidic pH (2 - 6) and at alkaline pH the activity of the plant extracts was reduced [12]. Some workers observed that bioactive compounds of plants were found to be stable over a wide range of pH values and temperatures [13].

Hence, this work has been done to determine the effect of various abiotic components (pH, storage, temperature, sunlight) on the activity and stability of the MIC of Petroleum ether extract, 100% alcoholic crude extract of *E. globulus* and bioformulation.

Materials and Methods

The extract and bioformulation were treated to varying physical parameters chosen for a specific time period, and then changes in the minimum inhibitory concentration of extract, activity of extract and bioformulation against the test organism were observed. A tubes containing minimum concentration of extract, bioformulation and without extract/bioformulation were maintained as a control in each set of experiments against *Alternaria solani*. In the present study, 100% alcohol crude extract and partially purified petroleum ether extract of *Eucalyptus globulus* leaf and best ratios (6,13,16,24) of bioformulation which is made by combining plant extracts, neem oil cake and Cow dung were used for the experiments.

Bioformulation ratio no. 6

Ingredients: 100% alcohol crude extract (1 ml): 100% neem oil cake (4 ml): 100% cow dung (5 ml).

Bioformulation ratio no. 13

Ingredients: 100% alcohol crude extract (2 ml): 100% neem oil cake (7 ml): 100% cow dung (1 ml).

Bioformulation ratio no. 16

Ingredients: Partially purified petroleum ether extract (5 ml): 100% neem oil cake (4 ml): 100% cow dung (1 ml).

Bioformulation ratio no. 24

Ingredients: Partially purified petroleum ether extract (2 ml): 100% neem oil cake (2 ml): 100% cow dung (6 ml).

These extracts and bioformulation were found with the best inhibitory activity.

Effect of sunlight

The effect of sunlight on the feasibility of extracts and bioformulation was studied according to this method. Aseptic vials containing 5 ml of 100% alcoholic crude extract, partially purified petroleum ether extract and bioformulation (ratio no. 6, 13, 16, 24) were kept

in sunlight for 15h and 30h [14]. After which effect on the efficacy of extract and bioformulation was assayed by two fold serial dilution method and poison food technique. In the poison food technique, 18 ml of molten PDA medium was poured into test tubes and then autoclaved. The molten sterilized medium along with 2 ml of extract /bioformulation was placed into petriplates after the solidification of the media, 6 mm inoculum disc of 7 day old culture of the fungus was aseptically inoculated upside down in the centre of the petriplate and incubated at 25 ± 2°C.

On the 7th day of incubation average diameter of the fungal colonies was measured and percent mycelia growth inhibition was calculated by the following formula given below:

% Mycelial growth inhibition=gc-gt/gc×100

Where,

gc = Growth of mycelia colony after incubation period in control set subtracting the diameter of inoculum's disc

gt = Growth of mycelia colony after incubation period in treatment set subtracting the diameter of inoculum's disc.

Effect of heat

Effect of heat on extract and bioformulation was assayed according to this method. Effect of dry heat was studied by exposing aseptic glass vials containing 100% alcoholic crude extract, partially purified petroleum ether extract and bioformulation (ratio no. 6, 13, 16, 24) to 40°C and 90°C for 4hrs in a hot air oven while in case of wet heat; extract and bioformulation were kept at 50°C and 100°C in a water bath for 4hrs [15]. Effect on the activity of extract and bioformulation was then assayed by two fold serial dilution method and poison food technique. One tube containing untreated extract as well as bioformulation (room temperature) was maintained as a control for comparison.

Effect of pH

Effect of varying pH i.e. 4, 7 and 9 on the efficacy of extract and bioformulation was studied by this method. The natural pH of extract and bioformulation is 7.0. 0.1 N HCl and 0.1 NaOH were used to change the pH to 4 and 9 respectively. Potato dextrose agar media was then added to tubes containing extract and bioformulation and the tubes were inoculated with *Alternaria solani*. Inoculated tubes were incubated at 27 ± 1°C for 72 hrs and observed for change in bioformulation activity and minimum inhibitory concentration of extract [16].

Effect of storage

Extract and bioformulation were stored at room temperature and change in their activity was assayed at regular intervals of 6 months up to 24 months by two fold serial dilution method and poison food technique [17].

Statistical analysis

All experiments were performed in triplicates (n = 3) and the data were presented as the mean ± standard deviation, student t- test and one way ANOVA. Significance was measured at $p < 0.001$.

Results and Observations

The results of the effect of different physical factors like light, temperature, pH and storage on extract and bioformulation of *E. globulus* leaf are given in table 1 to 8.

Table 1 and 2 shows that no change in the effect of petroleum ether extracts and bioformulations were seen when direct sunlight given for 15hrs and 30hrs. In 100% alcohol crude extract, 15hrs exposure had no effect as a zone of inhibition was 26.66 ± 0.01 mm observed but after 30h exposure, a slight decrease in activity and zone of inhibition was 30 ± 0.01 mm observed. Table 3 and 4 describe the effect of wet as well as dry heat on extract and bioformulation potential. Results indicate that there was no effect on the activity of 100% alcoholic crude extract and bioformulation ratio number 6, 13, 16 and 24 up to 50°C of wet heat and 40°C of dry heat however, heating at 100°C of wet heat and 90°C of dry heat for4 hrs resulted in a slight decrease in extract and bioformulation efficacy as a slight growth zone of test fungus was 22.6 ± 0.52 mm, 20.66 ± 0.52 mm, 20.33 ± 0.15 mm, 21 ± 0.23 mm for bioformulation no.6,13,16,and 24 respectively.

S. No.	Extracts	Antifungal activity of extracts under unexposed condition	Antifungal activity of extracts after 15 hrs exposure in sunlight	Antifungal activity of extracts after 30 hrs exposure in sunlight
1.	100% Alcoholic Crude extract	26.66 ± 0.57 (mm)	26.66 ± 0.57 (mm)	30 ± 0.57 (mm)
2.	Partially purified extract (PE)	2.5mg/ml	2.5mg/ml	2.5mg/ml
3.	Control (Without Extract)	72.33 ± 0.52 mm		
4.	p-value	0.88		
5.	Df	11		
6.	Significance	**		

Table 1: *Effect of sunlight exposure on crude and partially purified extract of eucalyptus globulus leaf extract against Alternaria solani.*

	Bioformulation Ratio Number	Antifungal activity of bioformulation under Unexposed Condition	Antifungal activity of bioformulation after 15 hrs exposure in sunlight	Antifungal activity of bioformulation after 30 hrs exposure in sunlight
1.	6	16.6 ± 0.52	16.6 ± 0.52	16.6 ± 0.52
2.	13	16.66 ± 0.52	16.66 ± 0.52	16.66 ± 0.52
3.	16	15.66 ± 0.57	15.66 ± 0.57	15.66 ± 0.57
4.	24	16.66. ± 0.57	16.66. ± 0.57	16.66. ± 0.57
5.	Control (Without Extract)	72.33 ± 0.52		
6.	p-value	0.0003		
7.	Df	47		
8.	Significance	****		

Table 2: *Effect of sunlight exposure on bioformulation against Alternaria solani.*

S. No.	Extract	Antifungal activity of extracts (Wet Heat)		Antifungal activity of extracts (Dry Heat)	
		50ºC	100ºC	40ºC	90ºC
1.	100% Alcoholic	26.66 ± 0.57 (mm)	30 ± 0.57 (mm)	26.66 ± 0.57 (mm)	28.33 ± 0.57 (mm)
2.	Partially Purified Petroleum ether	2.5 mg/ml	Slight growth	2.5 mg/ml	Slight growth
3.	Control (Without Extract)	72.33 ± 0.52			
4.	p-value	14			
5.	Df	0.58			
6.	Significance	***			

Table 3: Effect of heat on crude and partially purified extract of *Eucalyptus globulus* leaf against *Alternaria solani.*

S. No.	Bioformulation Ratio Number	Antifungal activity of bioformulation (Wet Heat)		Antifungal activity of bioformulation (Dry Heat)	
		50ºC	100ºC	40ºC	90ºC
1.	6	16.6 ± 0.57	20.6 ± 0.57	16.6 ± 0.57	19.66 ± 0.57
2.	13	16.66 ± 0.57	20.66 ± 0.57	16.66 ± 0.52	20.33 ± 0.57
3.	16	15.63 ± 1.57	23.33 ± 0.5	15.33 ± 0.57	20.33 ± 0.57
4.	24	16. ± 0.57	21.33 ± 0.57	16.33 ± 0.57	21 ± 0.00
5.	Control (Without Extract)	72.33 ± 0.52			
6.	p-value	0.76			
7.	Df	59			
8.	Significance	*			

Table 4: *Effect of heat on bioformulation against Alternaria solani.*

Table 5 and 6 shows the results and effect of pH on the potential of plant extract as well as bioformulation. The inhibitory effect was observed same (as observed in prior studies) at neutral and alkaline pH up to 9 but there was reduction in antifungal activity at acidic pH (pH-4) against *A. solani*.

S. No.	Extract	Antifungal activity of extracts Control (pH 7)	Antifungal activity of extracts pH 4	Antifungal activity of extracts pH 9
1.	100% Alcoholic crude	26.66 ± 0.01 (mm)	30 ± 0.01 (mm)	30 ± 0.01 (mm)
2.	Partially Purified Petroleum ether	2.5 mg/ml	Slight Growth	Slight Growth
3.	Control (Without Extract)	72.33 ± 0.52		
4.	p-value	0.42		
5.	df	11		
6.	Significance	***		

Table 5: *Effect of pH on crude and partially purified extract of Eucalyptus globulus leaf against Alternaria solani.*

S. No.	Bioformulation Ratio Number	Antifungal activity of bioformulation Control (pH 7)	Antifungal activity of bioformulation pH 4	Antifungal activity of bioformulation pH9
1.	6	16.6 ± 0.57	22.6 ± 0.57	22.6 ± 0.57
2.	13	16.66 ± 0.57	20.66 ± 0.57	20.66 ± 0.57
3.	16	15.33 ± 0.57	20.33 ± 0.57	20.33 ± 0.57
4.	24	16. ± 0.23	21 ± 0.23	21 ± 0.23
5.	Control (Without Extract)	72.33 ± 0.52		
6.	p-value	0.03		
7.	df	15		
8.	Significance	*****		

Table 6: *Effect of pH on bioformulation against Alternaria solani.*

Table 7 and 8 shows the result of the effects of storage of the extract and bioformulation at room temperature. Storage for 6 and 12 months had no changes in the antifungal potential of extract and bioformulation and the activity was observed the same as the fresh extract.

S. No.	Extract	Antifungal activity of Fresh Extract	Antifungal activity of extracts after 6 Months	Antifungal activity of extracts after 12 Months
1.	100% Alcoholic crude	26.66 ± 0.01 (mm)	26 ± 0.01 (mm)	26.66 ± 0.01 (mm)
2.	Partially Purified Petroleum ether	2.5 mg/ml	2.5mg/ml	2.5mg/ml
3.	Control (Without Extract)	72.33 ± 0.52		
4.	p-value	0.42		
5.	df	11		
6.	Significance	***		

Table 7: *Effect of storage on crude and partially purified extract of Eucalyptus globulus leaf against Alternaria solani.*

S. No.	Bioformulation Ratio Number	Antifungal activity of freshly prepared bioformulation	Antifungal activity of bioformulation after 6 Months	Antifungal activity of bioformulation after 12 Months
1.	6	16.66 ± 0.57	16.6 ± 0.57	16.6 ± 0.57
2.	13	16.66 ± 0.57	16.66 ± 0.57	16.66 ± 0.57
3.	16	15.33 ± 0.57	15.33 ± 0.57	15.33 ± 0.57
4.	24	16. ± 0.00	16. ± 0.00	16. ± 0.00
5.	Control (Without Extract)	72.33 ± 0.52		
6.	p-value	0.0004		
7.	df	15		
8.	Significance	****		

Table 8: *Effect of storage on bioformulation against Alternaria solani.*

Discussion

Now a day's bioformulations are very advantageous in the management of plant diseases as they have no adverse effects as compared to chemical fungicides. Bioformulation can only be viable when their stability and activity do not disturb by climatic factors because all these factors also play a major role in the development and action of the antimicrobial compounds present in the plants.

Results showed that no change was observed in antifungal activity of petroleum ether extract and bioformulation after exposure to direct sunlight which indicates that active principles of petroleum extract and bioformulation are light stable and do not undergo photo oxidation. 100% alcoholic crude extract also retained its antifungal potential up to 15 hrs exposure to sunlight. Probably sunlight exposure does not destruct the active molecules of petroleum ether extract of *Eucalyptus globulus* that possess antifungal potential.

Effect of heat on 100% alcohol crude extract and bioformulation ratio number 6 and 13 showed that the active principles can withstand the wet heat and dry heat up to 50°C and 40°C respectively. While continued exposure of extract with 100°C wet heat and 90°C dry heat destroyed its antifungal potential but it has no effect on petroleum ether extract and bioformulation ratio number 16 and 24. Some authors also concluded the same phenomenon in plant extracts when exposed to different temperatures ranging from 40°C to 60°C and 90°C [18,19].

The antifungal activity of extract and bioformulation of *Eucalyptus globulus* leaf was observed stable at pH 7 and 9 and reduction activity at pH 4 was observed. These results suggest that the active principles are showed better inhibitory activity at neutral pH. Some workers explained the presence of a high concentration of salt promotes the growth of microorganisms [20]. Some researchers showed that methanol extract of plant parts had a higher antioxidant activity at neutral pH [21]. Some authors have been reported that acidic pH increases the activity of phytoconstituents in plants [22,23].

Some authors have reported the antioxidant activity of plants is decreased at storage conditions [24]. Some workers showed that aqueous and ethanolic extracts of plants have similar antibacterial activity as the fresh extracts [25].

Different physical factors during storage accelerate the aging process of plant extract and chemical decomposition of active components which results in decrease in antifungal potential. These effects vary from species to species hence a degree of the changes in biological activity and chemistry of secondary metabolites due to storage is species-specific [26].

They also suggested that environmental conditions like temperature, light, heat as well as prolonged storage could lead the changes in chemical constituents or a decrease in the quantity of active constituent of extract but the degree of change in chemical constituents of plant extracts varies.

Conclusion

The Results suggested that the metabolites present in the *Eucalyptus globulus* leaf are highly susceptible to change in the physical environment. It concluded that extracts and bioformulation can be stored for 12 months, can be stable at alkaline pH, stand with exposure to sunlight and high temperature. Hence a small changes in physical conditions could improve its shelf life and can be used as biofungicides for controlling microorganisms.

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Authors Contribution Statement

Ms. Surbhi Mehta perceived the idea, carried out the research study, evaluated the results and drafted the manuscript. Prof. Kanika Sharma guided to Ms. Mehta in conducting this research study and also reviewed and approved the manuscript.

Conflicts of Interest

There are no conflicts of interest. As this is my original research work.

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