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# Bioformulation of antifungal herbal extract from *Curcuma caesia* Roxb. and *Ixora coccinea* L. against *Botrytis cinerea* Pers.

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## ABSTRACT

This paper represents the antifungal property of the crude rhizomatous extract of *Curcuma caesia* Roxb. and leaf extract of *Ixora coccinea* L. against *Botrytis cinerea*. First, the crude extract from two different plants were taken separately for antifungal screening and then both the extract used simultaneously to evaluate the better efficiency of the herbal formulation. The extract mixture shows higher efficiency in respect to antifungal potentiality than either of the plant when used solely. After that, I performed sequential solvent partitioning of the extract mixture to locate which fraction actually carries the antifungal one. Diethyl ether fraction was found to contain some bioactive phytochemical(s) that can be of ecofriendly use to control the spreading of this plant pathogen.

**Keywords:** Phytochemicals, Bioformulation, Extract mixture, *Curcuma caesia* Roxb., *Ixora coccinea* L., *Botrytis cinerea* Pers.

## INTRODUCTION

Plants produce an enormous array of secondary metabolites, and it is commonly reasoned that a significant part of this chemical diversity serves to protect plants against plant pathogens [1]. The problems of environmental pollution have stimulated investigations of alternative strategies for the control of pests and pathogens [2,3]. Apart from conventional fungicides and microbial biocontrol agents, plant products/extracts have found to be effective against a wide range of pathogens [4, 5]. Furthermore, plant product based biofungicides are systemic, specific in action, nonphytotoxic, cost effective and have poor environmental retention [6].

Zingiberaceae family constitutes a vital group of rhizomatous medicinal and aromatic plants characterised by the presence of volatile oils and oleoresins of export value. Generally, the rhizomes and fruits are aromatic, tonic and stimulant; occasionally they are nutritive. Some are used as food as they contain starch in large quantities while others yield an astringent and diaphoretic juice. The important genera coming under Zingiberaceae are *Curcuma*, *Kaempferia*, *Hedychium*, *Amomum*, *Zingiber*, *Alpinia*, *Elettaria* and *Costus*. *Curcuma caesia* Roxb. is a species with a blue rhizome (commonly called black turmeric), the color is much brighter and deeper blue than in *C. aeruginosa*. The leaves have a deep red-violet patch, which runs throughout the whole lamina. The rhizome extract of *C. longa* and *C. caesia* were effective against fungi like *Fusarium oxysporium*, *Aspergillus niger*, *A. nidulans*, *Alternaria solani* and bacteria like *Staphylococcus albus*, *E. coli*, and *Pseudomonas pyocyanea* etc. [7]. Beside this *C. longa* also possess some antifungal activity against some more pathogenic fungi like *Botrytis cinerea*, *Erysiphe graminis*, *Phytophthora infestans*, *Puccinia recondita*, *Pyricularia oryzae*, and *Rhizoctonia solani* [8]. The fungitoxic effects of alcoholic extracts of *C. longa* on *Fusarium udum*, which causes wilt disease of *Cajanus cajan* in *in vitro* and *in vivo*, were examined [9]. Leaf extract of *Citrus medica*, root extract of *Asparagus adscendens*, rhizome extracts of *C. longa* and *Zingiber officinale*, and bulb extract of *Allium sativum* inhibited up to 100% growth of *Fusarium udum* at higher concentrations. [10].

*Ixora coccinea* L. belongs to the family Rubiacae, is a common flowering shrub native to Asia including Bangladesh, Southern India, and Sri Lanka [11]. The flowers of *I. coccinea* are used in the treatment of dysentery, leucorrhoea, dysmenorrheal, haemoptysis, bronchitis and scabies [12]. The antimicrobial properties of the flower [13] and leaves [14] have also been reported.

*Botrytis cinerea* Pers. is an airborne plant pathogen with a necrotrophic lifestyle attacking over 200 crop hosts worldwide. Although there are fungicides for its control, many classes of fungicides have failed due to its genetic plasticity. It has become an important model for molecular study of necrotrophic fungi. *Botrytis cinerea* affects many plant species, although its most notable hosts may be wine grapes.

Present study involves the biological control of *Botrytis cinerea* using the extract mixture of *Curcuma caesia* and *Ixora coccinea*. Present study incorporates some additional evaluation of this mixture as an ecofriendly biocontrol agent of *Botrytis cinerea*.

## MATERIALS AND METHODS

**Preparation of Curcuma caesia rhizomatous extract**

2.5 kg shade dried rhizomes of *Curcuma caesia* Roxb. plants were powdered and extracted three times with 95% EtOH (each 500 ml, 48 h) at room temperature. The extract was evaporated under reduced pressure and a solid residual mass was obtained. The residual solid obtained was then subjected to sequential solvent partitioning for locating the antifungal property of the plant. As the crude extract was positive in antifungal assay, sequential solvent partitioning of the crude rhizomatous extract of *Curcuma caesia* and identification of the antifungal fraction was performed.

For the sake of convenience, the fraction obtained from *Curcuma caesia* was assumed as residue A.

**Preparation of *Ixora coccinea* leaf extract:**

2.5 kg shade dried leaves of *Ixora coccinea* were ground to a fine powder and then extracted in 5 liters of 50% aqueous ethanol at room temperature for 7 days. The extract was filtered and concentrated under reduced pressure and a solid, dark brown residual solid was obtained. The residual solid obtained was then subjected to sequential solvent partitioning for locating the antifungal property of the plant. As the crude extract was positive in antifungal assay, sequential solvent partitioning of the crude leaf extract of *Ixora coccinea* and identification of the antifungal fraction was performed.

For the sake of convenience, the fraction obtained from *Ixora coccinea* was assumed as residue B.

**Sequential solvent partitioning of residue A and residue B separately followed by identification of the antifungal fractions:**

Both the extract was filtered and the filtrate was charcoalised separately. The charcoalised fraction was separatey filtered repeatedly through Whatman No.42 filter paper and a clear brown filtrate was obtained. Two different filtrates were then separately partitioned over petroleum-ether (60-80ºc), diethyl ether and chloroform in two different sets. Each fraction was collected separately, dried over anhydrous sodium sulphate and was concentrated under reduced pressure. A brown residual solid mass was obtained in each case from residue A and B. Both the residual mass obtained from *C. caesia* and *I. coccinea* was again diluted separately in different container to produce five different concentrations (5 mg $ml^{-1}$, 10 mg $ml^{-1}$, 20 mg $ml^{-1}$, 40 mg $ml^{-1}$, and 80 mg $ml^{-1}$) and their antifungal property was evaluated.

Furthermore, five different dilution sets were prepared where 50% of the residual solid mass taken from *C. caesia* and rest 50% residual solid mass taken from *I. coccinea*. Here also the mixture was diluted in five different concentrations (5 mg $ml^{-1}$, 10 mg $ml^{-1}$, 20 mg $ml^{-1}$, 40 mg $ml^{-1}$, and 80 mg $ml^{-1}$) followed by evaluation of their antifungal property.

In each of the experiments a control set was maintained and MIC was calculated by proper measurement.

**Preparation of sample solution:**

The test solution was prepared by dissolving the dark brown residual mass in few drops of propylene glycol and then diluting with sterile water [15] in the concentration of 5 mg $ml^{-1}$, 10 mg $ml^{-1}$, 20 mg $ml^{-1}$, 40 mg $ml^{-1}$, and 80 mg $ml^{-1}$. Few drops of propylene glycol diluted with sterile water were used as control. All the dilutions were sterilized by filtration using membrane filter (0.02 µ pore size).

**Fungal strains:**

Pure cultures of *Botrytis cinerea* was procured from Microbial Type Culture Collection and gene bank, Chandigarh (MTCC /06/7/4260, Code- 359), India.

**Media preparation:**

The potato tubers were peeled off and weighed for about 250 g tubers were chopped into small pieces into the sterile conical flask. After boiling the supernatant were collected and dextrose (20g) with agar (20g- Microbiology Grade) to dissolve the ingredients. The pH of the medium was adjusted to 6.8 – 7.0. The total volume of the medium was adjusted to one liter. Finally, the medium was sterilized in autoclave at $121^0$C for 17 minutes.

**Antifungal assay:**

The fungal cultures used for this assay were 3 days old. Fungal suspension was prepared to contain approximately 1 x $10^6$ CFU $ml^{-1}$. An overnight broth culture was used to seed sterile molten PDA medium maintained at 45 °C. Small cylinders of agar were cut and scooped out using 7 mm sterile cork borer. 0.1 ml of test solutions of different above-mentioned concentrations were loaded separately to each cup with the help of micropipette. Propylene glycol with sterile water was loaded to maintain control. The petridishes were sealed with a strip of paraffin and incubated at 37ºC ± 1ºC for 24 hrs. The test solutions were allowed to diffuse into the agar from the cup. After incubation, the diameter of the inhibition zone around the well, were measured in cm every day after the fungal growth. The final measurement was taken when the control reached the full size within the petridish. If a culture grew in an irregular shape, two or more measurements were made and average was recorded. From the growth diameter of the fungal colony, the percentage of inhibition and the effective concentration for colony growth inhibition was calculated [16].

**Statistical analysis**

The observed values were expressed as mean ± standard deviation. All the values reflected in the table are statistically analyzed by paired t test with control and found significantly different at 5% level ($P<0.05$).

## RESULTS

**Antifungal screening of the crude extract of *Curcuma caesia*:**

The minimum inhibitory concentration (MIC) value of this sample against *Botrytis cinerea* was 10 mg/ml with 10.9 mm. inhibition zone formation **(Table 1 and Figure 1; IIa).** Inhibition percentage increases with the increase concentration of crude extract.

**Antifungal screening of the crude extract of *Ixora coccinea*:**

The minimum inhibitory concentration (MIC) value of this sample against *Botrytis cinerea* was 10 mg/ml with 12.7 mm. inhibition zone formation **(Table 1 and Figure 1; IIb).** Inhibition percentage increases with the increase concentration of crude extract.

**Antifungal screening of the extract mixture:**

Interestingly, the minimum inhibitory concentration (MIC) value of this sample against *Botrytis cinerea* was 5 mg/ml with 5.3 mm. inhibition zone formation **(Table 1 and Figure 1; IIc).** Inhibition percentage increases with the increase concentration of crude extract.

**Table 1:** Determination of antifungal activity of different extracts and the extract mixture

| Conc. (mg./ml) | Diameter of inhibition zone against *Botrytis cinerea* (in mm.) | | | |
|---|---|---|---|---|
| | Control | *Curcuma caesia* | Extract Mixture | *Ixora coccinea* |
| 80 | 0 | 18.7 ± 0.5 | 19.3 ± 0.3 | 17.1 ± 0.2 |
| 40 | 0 | 14.0 ± 0.3 | 17.9 ± 0.2 | 12.8 ± 0.2 |
| 20 | 0 | 12.1 ± 0.4 | 16.4 ± 0.4 | 13.4 ± 0.4 |
| 10 | 0 | 10.9 ± 0.3 | 15.4 ± 0.3 | 12.7 ± 0.3 |
| 5 | 0 | 0 (No inhibition) | 5.3 ± 0.2 | 0 (No inhibition) |

The observed values were expressed as mean ± standard deviation. The control sets show no inhibition zone. All the values reflected in the table are statistically analyzed by paired t test with control and found significantly different at 5% level ($P<0.05$).


**Figure 1:** Determination of antifungal activity of different extracts and the extract mixture

**IIa:** Inhibition zone of *Botrytis cinerea* with 10 mg/ ml of the extract of *Curcuma caesia*; **IIb:** Inhibition zone of *Botrytis cinerea* with 10 mg/ ml of the extract of *Ixora coccinea*; **IIc:** Inhibition zone of *Botrytis cinerea* with 5 mg/ ml of the extract mixture.

## DISCUSSION

From the above results it can be conclude that, the diethyl ether fraction of the extract mixture showed better antifungal potentiality against *Botrytis cinerea* over the extracts of *Curcuma caesia* or *Ixora coccinea*. At 10 mg/ml of dilution of the extract mixture, the inhibition zone increases up to 15.4 mm. which is far more than the sole effect of the extract of *Curcuma caesia* or *Ixora coccinea*. Table 1 also reflects that the inhibition zone produce by the extract mixture was very much larger even the inhibition zone produces by any extract alone at the same concentrations. Hence, the extract mixture can be identified as a better ecofriendly bioformulation of antifungal compounds.

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