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Bioformulation of an ecofriendly crop protectant using the herbal extract from *Curcuma aromatica* Salisb. and *Ixora coccinea* L.

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Abstract
This paper represents the antimicrobial potentialities of the crude rhizomatous extract of *Curcuma aromatica* Salisb. and leaf extract of *Ixora coccinea* L. First, the crude extract from two different plants were taken separately for antimicrbial 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 antimicrobial 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 antimicrobial one. Diethyl ether fraction was found to contain some bioactive phytochemical(s) that can be of ecofriendly crop protectant.

Keywords: phytochemicals, antimicrobial potentialities, crop protectant, extract mixture, diethyl ether fraction, active principles, *Curcuma aromatica* salisb., *Ixora coccinea* L.

1. Introduction
Curcuma aromatica Salisb. belongs to the family Zingiberaceae, is the vanharidra mentioned in the classical Ayurvedic texts. It is found distributed from China southwards to Sri Lanka. *C. aromatica* holds an important place in native perfumery and cosmetics. Fresh rhizome is yellowish and gives out a strong camphoraceous smell. Rhizome was valued as medicine, being regarded as a tonic and carminative, and was a valued toiletry and cosmetic item. In addition to *C. longa*, the genus includes other economically important species such as *C. aromatica*, used in medicine and in toiletry articles [1].
Ixora coccinea L. belongs to the family Rubiacae, is a common flowering shrub native to Asia including Bangladesh, Southern India, and Sri Lanka [2]. The flowers of *I. coccinea* are used in the treatment of dysentery, leucorrhoea, dysmenorrheal, haemoptysis, bronchitis and scabies [3]. The antimicrobial properties of the flower [4] and leaves [5] have also been reported.
Present study involves the evaluation of antimicrobial potentialities using the extract mixture of *Curcuma aromatica* and *Ixora coccinea*. This study also incorporates some additional evaluation of this mixture as an ecofriendly crop protectant that can be applied in living biological systems.

2. Materials and Methods
2.1 Preparation of *Curcuma aromatica* rhizomatous extract
2.5 kg shade dried rhizomes of *Curcuma aromatica* Roxb. plants was 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 antimicrobial property of the plant. As the crude extract was positive in antimicrobial assay, sequential solvent partitioning of the crude rhizomatous extract of *Curcuma aromatica* and identification of the antimicrobial fraction was performed.
For the sake of convenience the fraction obtained from *Curcuma aromatica* was assumed as residue A.

2.2 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 liter 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 antimicrobial property of the plant. As the crude extract was positive in antimicrobial assay, sequential solvent partitioning of the crude leaf extract of *Ixora coccinea* and identification of the antimicrobial fraction was performed.
For the sake of convenience the fraction obtained from *Ixora coccinea* was assumed as residue B.

2.3 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. aromatica* and *I. coccinea* was again diluted separately in different container

to produce different concentrations (500 µg/ ml to 100 µg/ ml) and their antimicrobial property was evaluated.
Furthermore five different dilution sets were prepared where 50% of the residual solid mass taken from *C. aromatica* and rest 50% (in mass/mass ratio) residual solid mass taken from *I. coccinea.* Here also the mixture was diluted in different concentrations (500 µg/ ml to 100 µg/ ml) followed by evaluation of their antimicrobial property.
In each of the experiments a control set was maintained and LC_{50} values were determined.

2.4 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 [6] in the concentration of 500 µg/ ml to 100 µg/ ml. 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).

2.5 Antimicrobial Assay
2.5.1 Evaluation of antibacterial activity
2.5.1.1 Microorganisms, culture media and incubating temperatures
The extracts including the extract mixture were individually tested against a panel of microorganisms including Gram negative *Serratia marcescens* (MTCC NO. 7298) incubated at 30^0C, *Erwinia herbicola* (MTCC NO. 3609) incubated at 37^0C, *Xanthomonas* sp. (MTCC NO. 7444) incubated at 30^0C and Gram positive *Arthrobacter chlorophenolicus* (MTCC NO. 3706) incubated at 28^0C. All the bacterial strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. The reference strains of bacteria were maintained on nutrient agar medium and LB medium slants at 4^0C with a subculture period of 30 days.

2.5.1.2 Composition of the media for bacterial cultures
Two kinds of medium was used here, Nutrient agar medium and LB agar medium. The medial compositions were elaborated in table 1.

Table 1: Medial composition for bacterial cultures

Constituents	Weight / Volume	1. Nutrient agar medium (pH 7.0)
Beef extract	1.0g	After adjusting the pH, volume of the medium was adjusted to 1 liter by adding double distilled sterile water. Nutrient broth medium has the same composition without agar.
Yeast extract	2.0g	
Peptone	5.0g	
NaCl	5.0g	
Agar	15.0g	
Tryptone	10.0g	2. LB agar medium (pH 7.0)
Yeast extract	5.0g	After adjusting the pH, volume of the medium was adjusted to 1 liter by adding double distilled sterile water. LB broth medium has the same composition without agar.
NaCl	10.0g	
Agar	15.0g	

2.5.1.3 Preparation of McFarland standard
The turbidity standard was prepared by mixing 0.5 ml of 1.75% (w/v) $BaCl_2.2H_2O$ with 99.5 ml of 1% H_2SO_4, $BaSO_4$ (v/v). The standard was taken in screw cap test tube to compare the turbidity. The bacterial culture of selected strains were grown for 48- 72 hours and subsequently mixed with physiological saline. Turbidity was corrected by adding sterile saline until McFarland 0.5 $BaSO_4$ turbidity standard 10^8 Colony Forming Unit (CFU) per ml was achieved. These inoculates were used for seeding of the nutrient agar medium, LB medium respectively.

2.5.1.4 Disc diffusion assay
1 mg of each sample(s) including the extract mixture (50% mass taken from residue A i.e. 0.5 mg, and rest 50% mass from residue B i.e. 0.5 mg) was separately dissolved in 1 ml of propylene glycol and then the volume was adjusted to 10 ml by adding sterile water. The ultimate concentration reaches to 10^2 µg/ ml and sterilized by filtration (0.22 µm filter). From the solution of each concentrated sample(s) final concentrations were made from 500 µg/ ml to 100 µg/ ml by adding sterile double distilled water. The sterile paper discs (6 mm diameter) were saturated with 10 µl of the solution of the respective sample(s) at a concentration of 500 µg/ ml to 100 µg/ ml and placed on the inoculated agar of 10^8 cfu/ml. Antibacterial tests were then carried out by disc diffusion method [7] using 100 µl of suspension containing 10^8 CFU/ml of bacteria on nutrient agar medium, LB medium respectively. Negative controls were prepared using propylene glycol. Gentamicin (10 µg/ disc) was used as positive reference standards to determine the sensitivity of each bacterial species tested. The inoculated plates were incubated at 30^0 C, 37^0 C, 30^0 C and 28^0 C respectively for 48 h, 24 h, 48 h and 72 h. Antibacterial activity was evaluated by measuring the zone of inhibition and the diameters of these zones were measured in millimeters against the test organisms.

2.5.1.5 Determination of minimum inhibitory concentration
The minimal inhibitory concentration (MIC) values were studied for the bacteria strains, being sensitive to the extracts and the extract mixture in disc diffusion assay. The inoculates of the bacterial strains were prepared from 24-72 hrs broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The extracts and the extract mixture was dissolved in sterile propylene glycol, were first diluted to the highest concentration (500 µg/ml) to be tested, and then serial dilutions were made in order to obtain a concentration range from 500 to 100 µg/ml in 10 ml sterile test tubes containing nutrient broth and LB broth medium respectively. MIC values of the extracts and the extract mixture against bacterial strains were determined based on a micro well dilution method as previously described [8]. The plate was covered with a sterile plate sealer and then incubated at appropriate temperatures for 24 - 72 h at 30^0 C, 37^0 C, 30^0 C and 28^0 C respectively.

Bacterial growth was determined by absorbance at 600 nm and confirmed by plating 10 µl samples, forming clear wells on nutrient agar medium or LB medium respectively. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. Each test in this study was repeated, at least, thrice.

2.5.2 Evaluation of antifungal activity

2.5.2.1 Fungal Strains

The reference strains used in the antifungal assays were: *Fusarium oxysporum*, *Botrytis cinerea* and *Rhizopus oryzae*. All the three fungal strains were procured from the plant biochemistry, molecular biology & advance plant physiology research laboratory, Department of Botany, University of Kalyani, India. All the test fungal strains were maintained on PDA medium (pH- 6.8) slants at 29^0C.

2.5.2.2 Composition of PDA medium

Potato infusion can be made by boiling of sliced (washed but unpeeled) potatoes in double distilled water for 30 minutes and then decanting or straining the broth through cheesecloth. Double distilled water is added such that the total volume of the suspension was made up to 1 liter. After that dextrose and agar powder was added. The medium was sterilized by autoclaving at 15 pounds per square inch (100 kPa) for 15 minutes. Detail composition of the PDA medium was elaborated in table 2.

Table 2: Composition of the PDA medium

Grams (G)/ milliliter (ml)	Ingredient
1000 ml	Water
200 G	Potatoes (sliced washed unpeeled)
20 G	Dextrose
20 G	Agar powder

2.5.2.3 Determination of minimum inhibitory concentration

Antifungal activity was screened by agar cup method [9-11]. All of the extracts and the extract mixture were tested against three plant pathogenic fungi like *Fusarium oxysporum*, *Botrytis cinerea*; and *Rhizopus oryzae* to access their antifungal nature. The PDA medium was poured in to the sterile petri plates and allowed to solidify under the sterile environment of the laminar air flow cabinet. The test fungal cultures were evenly spread over the media by sterile cotton swabs. Then wells of 9 millimeter were made in the medium using sterile cork borer. 100 µl of each sample having different concentrations were transferred into the separate wells which was made within the PDA medium. Plates containing the pure cultures of *Rhizopus oryzae* and *Botrytis cinerea* were allowed to incubated at 29^0C for 48-72 hours where as plates containing the pure cultures of *Fusarium oxysporum* takes incubation periods of 15-20 days at 29^0C. After the incubation period was over the plates were observed for formation of clear inhibition zone around the well indicated the presence of their antifungal nature. The zone of inhibition was recorded in millimeter scale. 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 an average was recorded. From the growth of the diameter of the fungal colony, the effective concentration for colony growth inhibition was calculated. All the above observations were taken in triplicate on each fungus/ sample concentration combinations. One control set was prepared identical to these and taking propylene glycol instead of different concentration combinations of sample solutions.

2.6 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 ($P<0.05$)

3. Results

3.1 Antibacterial activity screening of the crude extract of *Curcuma aromatica*

Antibacterial assay was also performed with residue A. Results in table number 3 indicate that this residue was positive in antibacterial assay and the MIC value was 232 µg/ml, 220 µg/ml, 353 µg/ml and 245 µg/ml for the bacterium *Serratia marcescens* (MTCC NO. 7298), *Erwinia herbicola* (MTCC NO. 3609), *Xanthomonas* sp. (MTCC NO. 7444) and *Arthrobacter chlorophenolicus* (MTCC NO. 3706) respectively (Table 3).

Hence, it may be cited that residue A have some antibacterial potentialities. This sample may be treated as a cost effective, eco-friendly, crop protectant against the common pathogens causing rot, blight and gall diseases in some vegetables as well as some crop plants.

Table 3: Assessment of antibacterial potentiality of the crude extract of *Curcuma aromatica* (MIC values are indicated within bracket)

Test sample	**Test bacterial strains**			
Acetylated derivative	**Diameter of inhibition zone in mm**			
Conc.(µg/ml)	***Serratia marcescens***	***Erwinia herbicola***	***Xanthomonas sp.***	***Arthrobacter chlorophenolicus***
500	19.3 ± 0.20	21.7 ± 0.21	18 ± 0.36	21.1 ± 0.15
400	16.1 ± 0.10	20.2 ± 0.20	13.2 ± 0.15	19 ± 0.10
353	15.9 ± 0.12	19.5 ± 0.25	11.3 ± 0.15 (MIC value)	14.1 ± 0.15
300	15.2 ± 0.06	17.2 ± 0.10	No activity	13.5 ± 0.20
253	14 ± 0.15	16.5 ± 0.31	No activity	12.5 ± 0.20
250	13.7 ± 0.12	14.9 ± 0.15	No activity	11.6 ± 0.25

245	12 ± 0.20	12.2 ± 0.21	No activity	10.2 ± 0.15 (MIC value)
232	10.5 ± .10 (MIC value)	8.5 ± .26	No activity	No activity
220	No activity	5.3 ± 0.15 (MIC value)	No activity	No activity
200	No activity	No activity	No activity	No activity
100	No activity	No activity	No activity	No activity

The observed values were expressed as mean ± standard deviation. Calculation was done with the help of spread sheet software Microsoft Excel 2010. All the values are statistically significant at (P<0.05)

3.2 Antifungal activity screening of the crude extract of *Curcuma aromatica*:

The minimum inhibitory concentration (MIC) values of residue A against *Fusarium oxysporum*, *Botrytis cinerea*, and *Rhizopus oryzae* were 253 µg /ml noted in every case (Table 4).

Table 4: Determination of the antifungal activity of the crude extract of *Curcuma aromatic*

Conc. (µg/ml)	Diameter of inhibition zone against different fungus (in mm.)		
	Botrytis cinerea	***Fusarium oxysporum***	***Rhizopus oryzae***
500	18.7 ± 0.5	18.7 ± 0.3	15.2 ± 0.2
400	17.0 ± 0.3	16.9 ± 0.2	14.9 ± 0.2
353	15.3 ± 0.4	16.4 ± 0.4	13.8 ± 0.4
300	12.9 ± 0.3	13.2 ± 0.3	12.3 ± 0.3
253	11.4 ± 0.4 (MIC value)	12.5 ± 0.4 (MIC value)	11.9 ± 0.2 (MIC value)
250	No inhibition	No inhibition	No inhibition
245	No inhibition	No inhibition	No inhibition
232	No inhibition	No inhibition	No inhibition
220	No inhibition	No inhibition	No inhibition
200	No inhibition	No inhibition	No inhibition
100	No inhibition	No inhibition	No inhibition

The observed values were expressed as mean ± standard deviation. The control sets shows 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).

3.3 Antibacterial activity screening of the crude extract of of *Ixora coccinea*

Antibacterial assay was also performed with residue B. Results in table number 4 indicate that this residue was positive in antibacterial assay and the MIC value was 353 µg/ml, 245 µg/ml, 220 µg/ml and 232 µg/ml for the bacterium *Serratia marcescens* (MTCC NO. 7298), *Erwinia herbicola* (MTCC NO. 3609), *Xanthomonas* sp. (MTCC NO. 7444) and *Arthrobacter chlorophenolicus* (MTCC NO. 3706) respectively (Table 5).

Hence, it may be cited that residue A have some antibacterial potentialities. This sample may be treated as a cost effective, eco-friendly, crop protectant against the common pathogens causing rot, blight and gall diseases in some vegetables as well as some crop plants.

Table 5: Assessment of Antibacterial activity screening of the crude extract of of *Ixora coccinea* (MIC values are indicated within bracket)

Test sample	Test bacterial strains			
Acetylated derivative	**Diameter of inhibition zone in mm**			
Conc. (µg/ml)	***Serratia marcescens***	***Erwinia herbicola***	***Xanthomonas sp.***	***Arthrobacter chlorophenolicus***
500	16 ± 0.36	19.1 ± 0.15	20.7 ± 0.21	18.3 ± 0.20
400	14.2 ± 0.15	16 ± 0.10	20 ± 0.20	17.1 ± 0.10
353	12.3 ± 0.15 (MIC value)	15.1 ± 0.15	19.1 ± 0.25	16.9 ± 0.12
300	No activity	13 ± 0.20	18.2 ± 0.10	16.2 ± 0.06
253	No activity	11.5 ± 0.20	17.5 ± 0.31	13 ± 0.15
250	No activity	11 ± 0.25	14.3 ± 0.15	12.7 ± 0.12
245	No activity	9.2 ± 0.15 (MIC value)	11.2 ± 0.21	12 ± 0.20
232	No activity	No activity	8 ±.26	10.5 ±.10 (MIC value)
220	No activity	No activity	6.3 ± 0.15 (MIC value)	No activity
200	No activity	No activity	No activity	No activity
100	No activity	No activity	No activity	No activity

The observed values were expressed as mean ± standard deviation.
Calculation was done with the help of spread sheet software Microsoft Excel 2010. All the values are statistically significant at (P<0.05)

3.4 Antifungal activity screening of the crude extract of *Ixora coccinea*

The minimum inhibitory concentration (MIC) values of residue B against *Fusarium oxysporum*, *Botrytis cinerea*, and *Rhizopus oryzae* were 245 µg /ml noted in every case (Table 6).

Table 6: Determination of antifungal activity of the crude extract of *Ixora coccinea*

Conc. (µg/ml)	Diameter of inhibition zone against different fungus (in mm.)		
	Botrytis cinerea	***Fusarium oxysporum***	***Rhizopus oryzae***
500	21.3 ± 0.4	21.1 ± 0.5	19.4 ± 0.2
400	17.7 ± 0.4	16.9 ± 0.2	18.3 ± 0.4
353	16.2 ± 0.2	14.3 ± 0.3	17.1 ± 0.3
300	15.3 ± 0.3	13.2 ± 0.2	15.9 ± 0.1
253	14.5 ± 0.4	12.4 ± 0.3	14.2 ± 0.3
250	12.9 ± 0.3	10.2 ± 0.4	13.5 ± 0.5
245	12.3 ± 0.3 (MIC value)	8.3 ± 0.3 (MIC value)	11.6 ± 0.4 (MIC value)
230	No inhibition	No inhibition	No inhibition
220	No inhibition	No inhibition	No inhibition
200	No inhibition	No inhibition	No inhibition
100	No inhibition	No inhibition	No inhibition

The observed values were expressed as mean ± standard deviation. The control sets shows 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).

3.5 Antibacterial activity screening of the extract mixture

Antibacterial assay was also performed with extract mixture. Results in table number 5 indicate that this sample was positive in antibacterial assay and the MIC value was 220 µg/ml for the bacterium *Serratia marcescens* (MTCC NO. 7298), *Erwinia herbicola* (MTCC NO. 3609), *Xanthomonas* sp. (MTCC NO. 7444) and *Arthrobacter chlorophenolicus* (MTCC NO. 3706) respectively (Table 7).

Hence, it may be cited that residue A have some antibacterial potentialities. This sample may be treated as a cost effective, eco-friendly, crop protectant against the common pathogens causing rot, blight and gall diseases in some vegetables as well as some crop plants.

Table 7: Assessment of antibacterial potentiality of the extract mixture (MIC values are indicated within bracket)

Test sample	Test bacterial strains			
Acetylated derivative	**Diameter of inhibition zone in mm**			
Conc. (µg/ml)	***Serratia marcescens***	***Erwinia herbicola***	***Xanthomonas sp.***	***Arthrobacter chlorophenolicus***
500	16 ± 0.36	20.7 ± 0.21	19.1 ± 0.15	18.3 ± 0.20
400	14.2 ± 0.15	20 ± 0.20	16 ± 0.10	17.1 ± 0.10
353	12.3 ± 0.15	19.1 ± 0.25	15.1 ± 0.15	16.9 ± 0.12
300	11.9 ± 0.20	18.2 ± 0.10	13 ± 0.20	16.2 ± 0.06
253	10.5 ± 0.20	17.5 ± 0.31	11.5 ± 0.20	13 ± 0.15
250	10 ± 0.25	14.3 ± 0.15	11 ± 0.25	12.7 ± 0.12
245	9.2 ± 0.15	11.2 ± 0.21	9.2 ± 0.15	12 ± 0.20
232	8.7 ± 0.15	8 ±.26	8.2 ± 0.15	10.5 ±.10
220	8.3 ± 0.15 (MIC value)	6.3 ± 0.15 (MIC value)	7.5 ± 0.15 (MIC value)	9.2 ± 0.15 (MIC value)
200	No activity	No activity	No activity	No activity
100	No activity	No activity	No activity	No activity

The observed values were expressed as mean ± standard deviation.

Calculation was done with the help of spread sheet software Microsoft Excel 2010. All the values are statistically significant at (P<0.05)

3.6 Antifungal activity screening of the extract mixture:

The minimum inhibitory concentration (MIC) values of the extract mixture against *Fusarium oxysporum*, *Botrytis cinerea* and *Rhizopus oryzae* were 230 µg /ml respectively (Table 8).

Table 8: Determination of antifungal potentialities of the extract mixture

Conc. (µg/ml)	Diameter of inhibition zone against different fungus (in mm.)		
	Fusarium oxysporum	***Botrytis cinerea***	***Rhizopus oryzae***
500	20.3 ± 0.4	19.1 ± 0.5	18.4 ± 0.2
400	16.7 ± 0.4	17.9 ± 0.2	17.5 ± 0.4
353	15.2 ± 0.2	15.3 ± 0.3	16.2 ± 0.3
300	14.8 ± 0.3	14.1 ± 0.2	15.3 ± 0.1
270	13.3 ± 0.4	13.1 ± 0.3	14.1 ± 0.3
250	12.1 ± 0.3	10.2 ± 0.4	12.5 ± 0.5
245	11.3 ± 0.3	9.9 ± 0.3	10.6 ± 0.4
230	10.9 ± 0.4 (MIC value)	9.4 ± 0.3 (MIC value)	8.7 ± 0.2 (MIC value)
220	No inhibition	No inhibition	No inhibition
200	No inhibition	No inhibition	No inhibition
100	No inhibition	No inhibition	No inhibition

The observed values were expressed as mean ± standard deviation. The control sets shows 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).

4. Discussion

From the above results it can be conclude that, the diethyl ether fraction of the extract mixture showed better antimicrobial potentiality over the extracts of *Curcuma aromatica* or *Ixora coccinea* used alone. At a minimal of dilution of the extract mixture, the MIC values of this sample were so much higher than the sole effect of the extract of *Curcuma aromatica* or *Ixora coccinea*. Hence, the extract mixture can be identified as a better ecofriendly bioformulation of antimicrobial compounds.

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