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Evaluation of Antimicrobial and Antifungal potential of (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine, isolated from *Curcuma caesia* Roxb.

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Abstract

The assessment of antimicrobial potentiality of (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine was the primary objective of this study. Assessment of its antimicrobial activity was performed using agar cup method and disc diffusion assay respectively. The bioactive compound was terpenoid in nature. The MIC values tested against different plant pathogenic fungi and bacteria. Antifungal assay showed the MIC values as 22 mg/ ml, 27 mg/ml, and 17 mg/ml for the test fungi *Botrytis cinerea*, *Fusarium oxysporum* and *Rhizopus oryzae* respectively. The MIC values of this mono-terpenoid was 267 µg/ml, 291 µg/ml, 345 µg/ml, and 467 µg/ml tested against the bacterium *Serratia marcescens*, *Erwinia herbicola*, Xanthomonas sp. and *Arthrobacter chlorophenolicus* respectively. This is the first report of the bioactive nature of (Z)-7-methoxy-1, 5-dihydrobenzo[c]oxepine. Due to its antimicrobial property it may function in plant defense or as an ecofriendly crop protectant or in ethnomedicinal purpose.

Keywords: *Curcuma caesia* Roxb., (Z)-7-methoxy-1,5-dihydrobenzo [c] oxepine, Antibacterial assay, Antifungal assay, Agar cup method, Disc diffusion assay, Crop Protectant.

Introduction

Now a day, solving the mystery of bioactive potentiality of natural product is one of the largest thrust areas of research in life science. Nature possesses all the disease curing agents (bioactive phytochemicals) that we need to reveal for our healthy life style. The use of Neem, Turmeric etc. in Indian tradition is found from ancient time, even when people do not exactly know the actual bioactive potentiality or mode of action of phytochemicals present there in. The genus Curcuma belongs to the family Zingiberaceae and contains 49 genera and 1400 species. In addition to *Curcuma longa*, *C. zedoaria* Rosc., *C. caesia* Roxb. and *C. xanthorrhiza* Roxb. are also minor sources of curcumin colour. The antimicrobial activities of methanolic extract were evaluated against several strains of bacteria and fungi.[1, 2, 3, 4, 5] The rhizome extract was effective against fungi *Fusarium oxysporium*, *Aspergillus niger*, *A. nidulans*, and *Alternaria solani* and bacteria *Staphylococcus albus*, *E. coli*, and *Pseudomonas pyocyanea*. Fungicidal activity of *C. longa* was also reported against *Botrytis cinerea*, *Erysiphe graminis*, *Phytophthora infestans*, *Puccinia recondita*, *Pyricularia oryzae*, and *Rhizoctonia solani*.[6] The experimental plant, *C. caesia* Roxb. (Black turmeric) of the family Zingiberaceae is a natural triploid, endemic and ethnomedicinally important plant.

Use of rhizomal extract of *C. caesia* Roxb. was very common among the tribal's of northeast India for its unique antimicrobial properties. In this paper we report for the first time the antimicrobial nature of (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine isolated from the shade dried rhizome of *Curcuma caesia* Roxb.

Materials and methods

Collection of Plant Material

Whole plant of *C. caesia* was collected in the month of July 2010 from experimental garden of Department of Botany, University of Kalyani, and identified in the Department of Botany, University of Kalyani, Nadia.

Extraction and isolation of crude secondary metabolite content

2.5 kg shade dried rhizomes of black turmeric plant was powdered and extracted three times with 1 liter of 95% EtOH at room temperature to give an extract of 479 gms. The extract was evaporated under reduced pressure and a solid residual mass was obtained. The above obtained residual sample was subjected to repeated preparative thin layer chromatography using different solvent systems, e.g solvent system 1. Methanol (5%): benzene (95%) and solvent system 2. Chloroform (60%): benzene (30%): acetic acid (10%). Three homogeneous spots were collected in solvent system 2, having Rf values of 0.87, 0.79 and 0.75 respectively. The sample with Rf value 0.75 was taken up for further study. This sample was positive in Liebermann's Burchard test[7] indicating its terpenoid nature and identified as (Z)-7-methoxy-1, 5-dihydrobenzo [c] oxepine.

Antifungal Assay

Preparation of Sample Solution

Approximately 1g of the sample isolated from *C. caesia* and transferred to a 20 ml volumetric flask. The compound was totally solubilised in 1 ml of propylene glycol and the total volume of the stock solution of the sample was adjusted to 10 ml by addition of sterile double distilled water. So the concentration of the stock solution of the sample was 100 mg/ml. By diluting the concentration of stock solution with the help of addition of sterile double distilled water different concentrations of the isolated sample like 25 mg/ml to 5 mg/ml was made. Propylene glycol with sterile double distilled water was loaded into the agar cup to maintain the control set. The test solutions were allowed to diffuse into the agar from the cup. All the dilutions were sterilised by filtration using membrane filter (0.02µ pore size).

Fungal Strains

The reference strains used in the antifungal assays were: *Fusarium oxysporum*, *Botrytis cinerea*; *Rhizopus oryzae*. All the fungal strains were procured from the Plant Biochemistry, Molecular Biology & Advance Plant Physiology Research Laboratory, Department of Botany, University of Kalyani, India. The test fungal strains were maintained on PDA medium (pH-6.8) slants at 29^0C.

Assessment of the Antifungal Potentialities

Antifungal activity was screened by agar cup method.[8-11] The isolated sample was 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.

Antibacterial assay

Microorganisms, Culture Media and their Incubating Environment

The isolated sample 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.

Composition of the Media

Details of composition of the media in which test microorganisms were grown are given in table 1.

Table 1: Composition of the media for test bacterium

Medium	Constituents	Weight / Volume	Description
A. 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	
B. LB agar medium (pH 7.0)	Tryptone	10.0g	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.
	Yeast extract	5.0g	
	NaCl	10.0g	
	Agar	15.0g	

Preparation of Mcfarland Standard

The turbidity standard was prepared by mixing 0.5 ml of 1.75% (w/v) $BaCl_2$.2H2O 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 inocula were used for seeding of the nutrient agar medium and LB medium respectively.

Disc Diffusion Assay

1 mg of the isolated sample 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 10^3 µg/ ml and sterilized by filtration (0.22 µm filter). The concentrations at 500 to 100 µg/ ml were taken in each case. The sterile paper discs (6 mm diameter) were saturated with 10 µl of the solution of the compound at a concentration of 500 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[12] using 100 µl of suspension containing 10^8 CFU/ml of bacteria on nutrient agar medium and 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 30^0C, 37^0C, 30^0C and 28^0C 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.[13-17]

Determination of Minimum Inhibitory Concentration

The minimal inhibitory concentration (MIC) values were studied for the bacteria strains, being sensitive to this compound in disc diffusion assay. The inocula of the bacterial strains were prepared from 24-72 hr broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The compound was dissolved in 1 ml of 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 compound against bacterial strains were determined based on a micro well dilution method as previously described.[18, 19] The plate was covered with a sterile plate sealer and then incubated at appropriate temperatures for 24 - 72 h at 30^0C, 37^0C, 30^0C and 28^0C 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.

Statistical Analysis

All the data represented in table number 2 and 3 obtained during in vitro experiments were expressed as mean ± standard deviation. Calculation was done with the help of spread sheet software Microsoft Excel 2010. * Indicates significance at ($P<0.05$).

Results

Assessment of Antifungal Potentialities

The minimum inhibitory concentration (MIC) values of (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine against *Botrytis cinerea*, *Fusarium oxysporum* and *Rhizopus oryzae* were 22 mg/ml, 27 mg/ml and 17 mg/ml repectively (Table 2).

Table 2: Assessment of antifungal potentialities of (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine

Serial number	Fungal strains	Concentration of the compound (MIC values)	Diameter of inhibition zone in mm
1	*Botrytis cinerea*	22 mg/ml	12±0.15
2	*Fusarium oxysporum*	27 mg/ml	7±0.21
3	*Rhizopus oryzae*	17 mg/ml	6.5±0.15

Assessment of Antibacterial Potentialities

Antibacterial assay was performed with (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine against four plant pathogenic bacterium and the MIC value was 267 µg/ml, 291 µg/ml, 345 µg/ml and 467 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).

Table 3: Assessment of antibacterial potentialities of (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine

Serial number	Bacterial strains	Concentration of the compound (MIC values)	Diameter of inhibition zone in mm
1	*Serratia marcescens*	267 µg/ml	5.4±0.15
2	*Erwinia herbicola*	291 µg/ml	9±0.20
3	*Xanthomonas* sp.	345 µg/ml	23±0.30
4	*Arthrobacter chlorophenolicus*	467 µg/ml	10±.20

Discussions

The isolated fraction (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine shows antifungal as well as antibacterial activities against some major plant pathogenic microbes. This terpenoid may be involved in the host defense mechanism of black turmeric. Isolation and characterization of this terpenoid including evaluation of its antimicrobial potentialities may also help us to use (Z)-7-methoxy-1, 5-dihydrobenzo[c] oxepine as a crop protactant.

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