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Evaluation of the bioactive potentialities of a diacetaldehyde terpenoid isolated from *Curcuma caesia* Roxb.

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Abstract

This paper represents evaluation of the bioactive potentialities of a diacetaldehyde terpenoid isolated from *Curcuma caesia* Roxb. The terpenoid was identified as (2Z,2'Z)-2,2'- (3aR,10aS)-1,3,5,8,9,9- hexamethyl- 1,2,3,3a-tetrahydrobenzo [f] azulene- 4,10 (5H,8H,9H,10aH) -diylidene) diacetaldehyde. Assessment of its antitumour activity, antifungal activity and antibacterial activity was performed using brine shrimp cytotoxicity assay, agar cup method and disc diffusion assay respectively. The antitumour, antifungal as well as antibacterial activity was promising and it showed 25 mg/ml (LC 50 value), (25 mg/ml, 67 mg/ml, 39 mg/ml), (235 µg/ml, 257 µg/ml, 210 µg/ml, 322 µg/ml) value respectively. This study represents the first report of bioactive nature of (2Z,2'Z)-2,2'- (3aR,10aS)- 1,3,5,8,9,9- hexamethyl-1,2,3,3a-tetrahydrobenzo [f] azulene- 4,10 (5H,8H,9H,10aH)- diylidene) diacetaldehyde isolated from plants.

Keywords: *Curcuma caesia* Roxb., (2Z,2'Z)-2,2'- (3aR,10aS)-1,3,5,8,9,9- hexamethyl-1,2,3,3a-tetrahydrobenzo [f] azulene- 4,10 (5H,8H,9H,10aH) -diylidene) diacetaldehyde, Antitumour assay, Antifungal assay, Antibacterial assay.

Introduction

Curcuma caesia Roxb. (Black turmeric) of the family Zingeberaceae is an important unexplored plant valued all over the Asia for its medicinal properties. So far eight natural products have been isolated and characterised from Curcuma caesia Roxb. like Borneol, Borneol acetate, 1,8-Cineole, α-Curcumene, γ-Curcumene, β-Elemene, (E)-β-Ocimene, ar-Turmerone etc.[1, 2] None of the phytochemicals isolated from *C. caesia* Roxb. have been accessed for their bioactive potentialities. This paper deals with the bioactive potentialities of (2Z,2'Z)-2,2'- (3aR,10aS)- 1,3,5,8,9,9- hexamethyl-1,2,3,3a-tetrahydrobenzo [f] azulene- 4,10 (5H,8H,9H,10aH)- diylidene) diacetaldehyde isolated from C. caesia Roxb. This is the first report of bioactive nature of this terpenoid.

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 was 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 *C. caesia* Roxb. 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 0.79 was taken up for further study. This sample was positive in Liebermann's Burchard test[3] and gave purple colour indicating terpenoid nature of the compound and had melting point of 78^0C.

Antitumour assay

Brine Shrimp Cytotoxicity assay was done following the method of B. N. Meyer et al.[4-7] Brine shrimp eggs were hatched in a shallow rectangular dish (22×32×12 cm), one third of which was filled with saline water. An aluminium divider with several 2 mm holes was clamped in the dish to make to unequal compartments. The eggs (50 mg) were sprinkled into the larger compartment which was darkened while the smaller compartment was illuminated. The set was maintained at 30^0C- 32^0C and after 48 hours the phototropic nauplii was collected by pipette from the lighter side, having been separated by the divider from their shells.

The shrimps were transferred to each sample vial using a 23 cm disposable pipette and saline water was added to adjust the volume to 5 ml. The nauplii could be counted in the stem of the pipette against a lighted background. A drop of dry yeast suspension (3 mg in 5 ml of saline water) was added as food to each vial. The vials were maintained under illumination at room temperature. Surviving shrimps was counted after every 3 hours up to 24 hours and the percentage of death at each dose and control was determined.

$$\text{Death (\%)} = \text{test-control} \times 100/\text{control}$$

Four replicates were prepared for each dose level and after 24 hours LC_{50} values were determined.

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 50 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 potentiality

Antifungal activity was screened by agar cup method.[8-12] The isolated samples and their different derivatives 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 millimetres 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 millimetre 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

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.

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 inocula were used for seeding of the nutrient agar medium and LB medium respectively.

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.

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 103 µg/ ml and sterilized by filtration (0.22 µm millipore 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[13] 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 millimetres against the test organisms.[14-18]

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.[19, 20] 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 1, 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 antitumour assay

The isolated sample, named (2Z,2'Z)- 2,2'-(3aR,10aS)- 1,3,5,8,9,9-hexamethyl- 1,2,3,3a-tetrahydrobenzo [f] azulene-4,10 (5H,8H,9H,10aH)-diylidene) diacetaldehyde was positive in brine shrimp assay [5, 6, 7, 8] and the LC_{50} value was 25 mg/ml (Table 2).

Table 2: Antitumour assay of (2Z,2'Z)-2,2'- (3aR,10aS)- 1,3,5,8,9,9-hexamethyl- 1,2,3,3 a- tetrahydrobenzo [f] azulene-4,10 (5H,8H,9H,10aH)- diylidene) diacetaldehyde

Concentration (mg/ml)	**No. of survivals after**									**LC $_{50}$**
	0 hrs	3 hrs	6 hrs	9 hrs	12 hrs	15 hrs	18 hrs	21 hrs	24 hrs	
0	18.0 ± 0.0	18.0 ± 0.0	18.0 ± 0.0	18.0 ± 0.0	18.0 ± 0.0	18.0 ± 0.0	18.0 ± 0.0	18.0 ± 0.0	18.0 ± 0.0	
1	18.0 ± 0.0	16.0 ± 0.2*	15.7 ± 0.2*	14.5 ± 0.0	14.0 ± 0.3*	12.6 ± 0.4*	12.2 ± 01*	11.5 ± 0.4*	11.1 ± 0.5*	
10	18.0 ± 0.0	16.0 ± 0.02*	15.0 ± 0.5*	14.4 ± 0.3*	14.0 ± 0.2*	12.3 ± 0.2*	12.0 ± 0.6*	11.3 ± 0.3*	10.6 ± 0.1*	
25	18.0 ± 0.0	15.9 ± 0.4	15.3 ± 0.3*	14.5 ± 0.3*	14.2 ± 0.6*	12.6 ± 0.1*	11.3 ± 0.2*	11.0 ± 0.2*	9.0 ± 0.3*	25mg/ml
50	18.0 ± 0.0	15.3 ± 0.2*	14.8 ± 0.3*	14.1 ± 0.6*	13.7 ± 0.2*	12.1 ± 0.7*	10.3 ± 0.3*	9.4 ± 0.8*	8.2 ± 0.1*	
75	18.0 ± 0.0	14.2 ± 0.5*	14.0 ± 0.2*	13.1 ± 0.2*	12.6 ± 0.6*	11.8 ± 0.1*	9.8 ± 0.3*	8.3 ± 0.7*	6 ± 0.5*	
100	18.0 ± 0.0	13.4 ± 0.3*	11.7 ± 0.3*	10.3 ± 0.6*	9.2 ± 0.2*	8.3 ± 0.1*	7.1 ± 0.7*	6.3 ± 0.4*	5.2 ± 0.2*	

125	18.0 ±0.0	12.6 ±0.5*	11.5 ±0.2*	9.9 ±0.1*	8.1 ±0.6*	7.7 ±0.3*	6.2 ±0.2*	4.4 ±0.2*	3 ± 0.5*	
250	18.0 ± 0.0	11.2 ± 0.2*	10.5 ± 0.1*	9.1 ± 0.3*	7.3 ± 0.3*	5.9 ± 0.5*	5.3 ± 0.3*	4.1 ± 0.3*	2 ± 0.1*	
500	18.0 ± 0.0	10.3 ± 0.4*	9.7 ± 0.3*	8.2 ± 0.3*	6.1 ± 0.5*	5.1 ± 0.1*	4.7 ± 0.2*	3.5 ± 0.5*	2.1 ± 0.6*	
750	18.0 ± 0.0	10.2 ± 0.2*	9.2 ± 0.2*	7.2 ± 0.1*	5.3 ± 0.3*	4.1 ± 0.4*	3 ± 0.4*	1.9 ± 0.3*	0	
1000	18.0 ± 0.0	9.5 ± 0.5	8.3 ± 0.5*	6 ± 0.2*	4.6 ± 0.1*	3.3 ± 0.3*	1.2 ± 0.4*	0	0	

The observed Values were expressed as mean ± standard deviation.
Calculation was done with the help of spread sheet software Microsoft Excel 2010.
* Indicates significance at ($P<0.05$)

Assessment of antifungal potentialities

The minimum inhibitory concentration (MIC) values of (2Z,2'Z)-2,2'- (3aR,10aS)- 1,3,5,8,9,9-hexamethyl- 1,2,3,3 a- tetrahydrobenzo [f] azulene- 4,10 (5H,8H,9H,10aH)- diylidene) diacetaldehyde against Fusarium oxysporum, Botrytis cinerea and Rhizopus oryzae were 25 mg/ml, 67 mg/ml and 39 mg/ml repectively (Table 3).

Table 3: Antifungal potentialities of (2Z,2'Z)-2,2'- (3aR,10aS)- 1,3,5,8,9,9-hexamethyl- 1,2,3,3 a- tetrahydrobenzo [f] azulene-4,10 (5H,8H,9H,10aH)- diylidene) diacetaldehyde

Fungal strains	**Concentration of the compound (MIC values)**	**Diameter of inhibition zone in mm**
Fusarium oxysporum	25 mg/ml	2.9±0.15
Botrytis cinerea	67 mg/ml	9.5±0.20
Rhizopus oryzae	39 mg/ml	13±0.30

Assessment of antibacterial potentialities

Antibacterial assay was performed with (2Z,2'Z)-2,2'- (3aR,10aS)- 1,3,5,8,9,9- hexamethyl-1,2,3,3 a- tetrahydrobenzo [f] azulene- 4,10 (5H,8H,9H,10aH)- diylidene) diacetaldehyde against four plant pathogenic bacterium and the MIC value was 235 µg/ml, 257 µg/ml, 210 µg/ml and 322 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 4).

Table 4: Antibacterial potentialities of (2Z,2'Z)-2,2'- (3aR,10aS)- 1,3,5,8,9,9-hexamethyl- 1,2,3,3 a- tetrahydrobenzo [f] azulene-4,10 (5H,8H,9H,10aH)- diylidene) diacetaldehyde

Bacterial strains	**Concentration of the compound (MIC values)**	**Diameter of inhibition zone in mm**
Serratia marcescens	235 µg/ml	9±0.47
Erwinia herbicola	257 µg/ml	6±0.35
Xanthomonas sp.	210 µg/ml	5±0.15
Arthrobacter chlorophenolicus	322 µg/ml	3.8±0.21

Discussions

Evaluation of bioactive potentialities

The isolated fraction (2Z,2'Z)- 2,2'- (3aR,10aS)- 1,3,5,8,9,9- hexamethyl- 1,2,3,3a -tetrahydrobenzo [f] azulene- 4,10 (5H,8H,9H,10aH) - diylidene) diacetaldehyde shows antitumour, antifungal as well as antibacterial activities.

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