Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 38-41
Received: 27-07-2017
Accepted: 28-08-2017

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Antimicrobial, membrane stabilizing and thrombolytic activities of ethanolic extract of *Curcuma zedoaria* Rosc. Rhizome

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Abstract
The present study was undertaken to evaluate the inherent anti-inflammatory, thrombolytic and antimicrobial potential of ethanolic extract of the rhizome of *Curcuma zedoaria* (EECZR). *In vitro* anti-inflammatory activity was uncovered by using human erythrocyte membrane stabilization and egg albumin denaturation method. *In vitro* thrombolytic activity was investigated and compared with streptokinase. Antimicrobial activity was evaluated by using disc diffusion method. EECZR dose dependently showed a potent anti-inflammatory activity. In addition, thrombolytic activity was found to be 28.46±4.16%, 9.67±6.37 and 65.96±4.25% for EECZR, normal saline and streptokinase (a positive control) respectively. This extract also showed potent antibacterial activity against eight pathogenic bacteria and produced inhibition zone ranging from 10 to 15 mm. The minimum inhibitory concentrations (MICs) of the ethanol extract was found to be in the range of 64 ~ 256 µg/ml. Our results suggest that ethanol extract of *Curcuma zedoaria* rhizome has strong anti-inflammatory, thrombolytic and antimicrobial activities.

Keywords: *Curcuma zedoaria*, rhizome, anti-inflammatory, thrombolytic, antibacterial

1. Introduction
Mortality caused infectious diseases has been being considered as one of the major health related issues throughout the world, primarily in the developing countries for many decades [1]. Regularly used antibiotics invented in the last decades are being increasingly less effective or inactive due to resistant properties of microbes or being indifferent in use. Synthetically manufactured antibiotics are also being inactive. For this infectious agents are recurrently being exposed to human body and create different fatal diseases [2]. Plants contain huge amount of related or different metabolites that are being traditionally used as drugs for many years. In addition formulated form of plant metabolites are also being used to cure different fatal diseases. The antimicrobial activity of various plant extracts have been reported by a number of researchers throughout the world [3].

Inflammation, a complex localized response of vascular tissue to foreign substances characterized by redness, warmth, swelling and pain, is a normal protective response to tissue injury [4, 5]. But prolonged inflammation is harmful for health as it produces various diseases like rheumatoid arthritis, atherosclerosis, hey fever, ischemic heart diseases etc. Some non-steroidal drugs are used for the management of inflammatory condition which may cause gastric bleeding, ulceration, bone marrow disturbance, kidney and liver dysfunction [6, 7]. For these reason, there is a need to find alternative and natural drugs having less or no side effects to use for oxidative stress and chronic inflammatory disease.

The plant *Curcuma zedoaria,* popularly known as Sothi (Bengali), belongs to the family Zingiberaceae and has a wide spread occurrence in Bangladesh, India, China, and South East Asia [8]. Rhizome is the most important part of this plant and is used to treat bronchities, asthma, leucoderma, amenorrhea-abdominal pain and rheumatic pain etc. Curcuminoids, turmerone and sesquiterpenoids isolated from *C. zedoaria* show cytotoxicity against human ovarian cancer OVCAR-3 cells and inhibit LPS-induced prostaglandin E_2 and nitric oxide production in cultured mouse macrophage cell RAW 264.7 [9-11]. Extracts of *C. zedoaria* have been found to have a moderate antimutagenic activity against benzo[*a*] pyreneb and have significantly reduced the number of metastatic surface nodules in the lung and extended life span of B16 melanoma cells [12, 13]. Data on anti-inflammatory, thrombolytic and antimicrobial properties of this plant is insufficient. Therefore, the present study was undertaken to explore the anti-inflammatory, thrombolytic and antibacterial activities of ethanolic extract of

Curcuma zedoaria rhizome.

2. Materials and methods

2.1 Plant materials

Rhizomes of *Curcuma zedoaria* (Family: Zingiberaceae) were collected from the hilly areas of Chittagong, Bangladesh and identified by an expert taxonomist, Dr. Mohammed Yusuf, Bangladesh Council of Scientific and Industrial Research, Chittagong, where a voucher specimen (No. 38765) of this collection was deposited for further analysis.

2.2 Extraction

200g of dried powdered rhizome of *Curcuma zedoaria* were placed in an amber colored extraction bottle and soaked with 500 ml of ethanol. The extraction bottle and its contents were then sealed and allowed for 7 days with occasional shaking and stirring. The whole mixture was filtered through cotton followed by Whatman No. 1 filter papers and was then concentrated with a rotary evaporator under reduced pressure at 40ºC to have ethanol extracts (4.5g).

2.3 Evaluation of *in vitro* anti-inflammatory activity

2.3.1 Membrane stabilizing activity

Two methods namely hypotonic solution-induced and heat-induced human erythrocyte haemolysis were applied to illuminate the membrane stabilizing activity [14, 15].

Hypotonic solution- induced haemolysis

To perform this test, 0.5 ml of RBC suspension and 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) were placed in a test tube which is followed by the addition of 1ml of extract/standard with various concentrations. A mixture containing all of the materials mentioned above except the sample/standard was taken as control. After successive incubation (for 10 minutes) and centrifugation (at 3000rpm for 10 min) the absorbance was taken at 540 nm. Acetyl salicylic Acid treating similarly was used as reference standard.

Heat- induced haemolysis

5 ml aliquots (containing isotonic buffer) of the extract were kept into two sets of centrifuge tubes. Same amount of vehicle was served as control. 30 µl of RBC suspension was mixed to each centrifuge tube. Then, incubation of one pair mixed to each centrifuge tubes was carried out at 54ºC for 20 min in a water bath. The temperature of another pair was maintained at 0-5^0 C. The mixture of each tube was then centrifuged at 1300 rpm for 5 min. Absorbance was recorded of the supernatant at 540 nm. Acetyl salicylic Acid treating similarly was used as reference standard.

2.3.2 Egg albumin denaturation assay

The method described by Dapurkar *et al* was followed to perform the egg albumin denaturation assay [16] in which 5ml of reaction mixture was made by taking 2 ml of sample extract with various concentrations, 0.2 ml of albumin isolated from fresh hen's egg and 2.8 ml of phosphate buffered saline (PBS, pH 6.4). Same amount of doubled-distilled water was taken as control. Incubation of each mixture was carried out at 37°C±2 for 15 min followed by heating at 70°C for 5 min. The mixtures were cooled and their absorbance was recorded at 660 nm. Acetyl salicylic Acid treating similarly was used as reference standard.

2.4 Determination of thrombolytic activity

The thrombolytic activity of EECZR was evaluated by the method developed by Daginawala (2006) [17] and slightly modified by Kawsar *et al*. (2011) [18] using streptokinase (SK) as the standard**.** Commercially available lyophilized Altepase (Streptokinase) vial (Beacon pharmaceutical Ltd.) of 15, 00,000 I.U., was collected and 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock from which100µL (30,000 I.U) was used for *in vitro* thrombolysis. Blood (n=6) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1mL of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

2.5 Antimicrobial activity

Antibacterial activity of the sample was examined against eight pathogenic bacteria (four Gram positive and four Gram negative) and three pathogenic fungi by using disc diffusion method [19]. Pure culture of bacteria [*Bacillus cereus* QL 29, *Bacillus megaterium* QL 38, *Staphylococcus aureus* ATCC25923, *Sarcina lutea* QL 166, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* AM 16406, *Shigella boydii* ATCC13147, and fungi [*Candida albicans*, *Aspergillus niger*, *Sacharomyces cerevaceae*] were collected from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. The sample solution of different concentrations was prepared and then applied to sterile disc (5 mm diameter, filter paper), followed by drying off the solvent in an aseptic hood. To compare the activity with standard antibiotics, Amphicillin discs (30µg/disc) and blank discs (impregnated with the corresponding solvents only) served as positive control and negative control, respectively.

2.6 Minimum inhibitory concentration (MIC)

Serial dilution technique was employed to determine the MIC of ethanol extract [20] against *Bacillus cereus* QL 29, *Sarcina lutea* QL 166, *Escherichia coli* ATCC 25922 and *Salmonella typhi* AM 16406.

3. Result and Discussions

3.1 Anti-inflammatory activity assay

RBC membrane shares many properties with lysosomal membrane. Phospholipases released from RBC that lead to the generation of inflammatory mediators can be inhibited by compounds having membrane stabilizing properties [21]. Table 1 showed that EECZR significantly protected the lysis of human erythrocyte membrane induced by hypotonic solution and heat induced haemolysis, as compared to the standard acetyl salicylic acid. In hypotonic solution and heat induced conditions, the EECZR inhibited 66.39% and 71.59% haemolysis of RBCs, respectively as compared to 74.93% and 95.96% inhibited by acetyl salicylic acid, respectively (500 µg/ml). Hypotonic solution induced hemolysis assay showed that the extract effectively protect the membrane of RBC. The extract also inhibited the heat induced hemolysis of RBCs.

It has been reported that protein denaturation is one of the causes of rheumatic arthritis. Many drugs used for the treatment of inflammatory conditions have been reported to prevent thermally induced protein denaturation [21]. In Egg Albumin denaturation method, at concentration of 100, 200, 300, 400 and 500 µg/ml EEACR showed 36.06%, 41.19%, 55.99%, 64.32% and 73.23% inhibition of protein denaturation, while at the same concentrations, standard ASA

showed 41.01%, 58.97%, 65.33%, 71.91% and 85.87% inhibition, respectively (Table 1). From the result of the present study, the extract had shown considerable anti-inflammatory activity.

3.2 Thrombolytic activity

In an attempt to discover a cardio-protective drug from natural sources, EECZR were assessed for thrombolytic activity [22] and the mean difference in clot lysis percentage between normal saline and EECZR was found statistically very significant ($p<0.01$). At a concentration of 100µg/mL streptokinase, EECZR and normal saline showed 65.96±4.25%, 47.08±5.11% and 6.67±2.37% thrombolytic activity, respectively (Table 2).

3.3 Antimicrobial activity of EECZR

As shown in the Table 3, the ethanol extract exhibited potent antibacterial activity with zone of inhibition of 15 mm each against *E. coli* and *Salmonella typhi*, followed by *Shigella boydii* (13 mm), *Bacillus cereus* (12 mm), *Bacillus megaterium* (12 mm), *Staphylococcus aureus* (11 mm), *Sarcina lutea* (10 mm) and *Pseudomonas aeruginosa* (11 mm). Out of the three species of fungi, *Aspergillus niger* and *Candida albicans* showed the inhibitory zone of 9 and 10 mm each while *Sacharomyces cerevaceae* showed no zone of inhibition. We also estimated the MIC values of the extract under this study against four bacteria (two gram positive and two gram negative bacteria). The lowest MIC value observed for ethanol extract against *Escherchia coli* was 64 µg/ml (Table 4). In previous studies, the main six components of ethanol extract of *Curcuma zedoaria* identified by GC-MS have been reported to possess antimicrobial potency against different bacterial strains [23-26]. In our study ethanol extract of *Curcuma zedoaria* showed considerable broad-spectrum antibacterial activity against some pathogenic bacteria indicating the probable application of this extract to manage infectious conditions.

4. Conclusion

The present study concludes that ethanol extract of *Curcuma zedoaria* rhizome possesses strong anti-inflammatory, thrombolytic and antibacterial activities which is comparable to the commercial anti-inflammatory, thrombolytic and antibacterial agents and may be helpful for the treatment of various human diseases. In future, investigation should be carried out regarding the identification and characterization of active principles of *Curcuma zedoaria* rhizome.

Table 1: Anti-inflammatory assay of ethanol extract of *Curcuma zedoaria* rhizome

Concentration (µg/ml)	Percentage inhibition					
	Hypotonic solution induced hemolysis		Heat induced hemolysis		Protein denaturation assay	
	EECZR	ASA	EECZR	ASA	EECZR	ASA
100	32.12	36.06	48.32	48.75	36.06	41.01
200	39.95	44.29	53.39	59.30	41.19	58.97
300	47.23	51,30	59.47	74.52	55.99	65.33
400	52.35	63.09	64.53	86.84	64.32	71.91
500	66.39	74.93	71.59	95.96	73.23	85.87

Table 2: Thrombolytic activity of ethanol extract of *Curcuma zedoaria* rhizome

Sample	Percentage (%) of lysis
EECZR	47.08±5.11
Streptokinase (Standard)	65.96±4.25
Normal saline	6.67±2.37

Table 3: Antibacterial activity of ethanol extract of *Curcuma zedoaria* rhizome

Test Bacteria	Diameter of zone of inhibition (mm) of the ethanol extract		Standard antibiotic Nalidixic acid
	400 µg/disc	800 µg/disc	(30µg/disc)
Bacillus megaterium	10 ± 0.5	12 ± 0.2	24± 0.8
Bacillus cereus	10 ± 0.8	12 ± 1.6	24 ± 0.5
Sarcina lutea	7.0±0.7	10±0.9	20±0.6
Staphylococcus aureus	9.0±0.4	11±0.6	25±0.3
Shigella boydii	10 ± 0.9	13 ± 0.5	21 ± 0.3
Pseudomonas ariginosa	12±0.5	15±0.3	22±0.4
Escherichia coli	12 ± 0.5	15± 0.7	28 ± 0.4
Salmonella typhi	12 ± 1.9	14 ± 1.0	25± 0.5
Test Fungi			
Sacharomyces cerevaceae	-	-	24±0.4
Candida albicans	7.0±0.7	9.0±0.9	25± 0.5
Aspergillus niger	8.0±0.8	10±0.5	20±0.3

Data are expressed as mean ± S.E.M (Standard error of mean)

Table 4: The result of MIC of tested ethanol extract of *Curcuma zedoaria* rhizome

Name of bacteria	MIC (µg/ml)
Bacillus cereus	128
Sarcina lutea	256
Escherichia coli	64
Salmonella typhi	128

Acknowledgements

The authors wish to thank Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh for providing pathogenic microorganisms for the study.

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