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Suraj Shakya
Nobel College,
Faculty of Health Science,
Department of Pharmacy,
Kathmandu-Nepal.

Anil Giri
Nobel College,
Faculty of Health Science,
Department of Pharmacy,
Kathmandu-Nepal.

Pratima Chhetri
Nobel College,
Faculty of Health Science,
Department of Pharmacy,
Kathmandu-Nepal.

Sujana Shakya
Nobel College,
Faculty of Health Science,
Department of Pharmacy,
Kathmandu-Nepal.

Correspondence:
Suraj Shakya
Nobel College,
Faculty of Health Science,
Department of Pharmacy,
Sinamangal P.O. Box: 10420,
Kathmandu, Nepal.
surajshakya_ptn@hotmail.com
Contact no.: +977-9841475683

Preliminary investigation of different extracts of *Curcuma caesia* for its antibacterial effects and cytotoxic activity

Suraj Shakya, Anil Giri, Pratima Chhetri, Sujana Shakya

Abstract
The present study is carried out with the different (Petroleum ether, Diethyl ether, Chloroform and Methanol) extracts of dried rootstocks and rhizome of *Curcuma caesia*. Traditional use of it as liver tonic, antifungal, pain reliever as well as anthelmintic inspired us to investigate the antimicrobial and cytotoxic activity of this plant. Preliminary phytochemical screening revealed the presence of alkaloids, terpenoids, flavonoids, deoxysugars as well as C-glycosides. The present study revealed that the tannin was absent in all extract of *C. caesia*, which may be a possible reason for not showing any antibacterial activity. Also the brine shrimp bioassay revealed that petroleum ether extract, diethyl ether extract, chloroform extract and methanol extract showed LC_{50} value of 18.923 mcg/ml, 1.086 mcg/ml, 45.289 mcg/ml and 100 mcg/ml respectively and since the LC_{50} values less than 1000 mcg/ml indicate the cytotoxic property of extracts, all the extracts of the plant were biologically active.

Keywords: *Curcuma caesia*, phytochemical screening, Antibacterial activity, cytotoxic activity.

1. Introduction
Turmeric usage dates back nearly 4000 years, to the Vedic culture in India, when turmeric was the principal spice and also of religious significance. It is employed in some Hindu rituals, where the yellow colour symbolized the sun. The genus name *Curcuma* likens turmeric to saffron, the most relevant yellow plant dye in the Ancient World [1].
Curcumas are the unimaginable world of hidden gingers, which get its name from some of the varieties because they bear their flowers on short stalks amid its foliage [2]. *Curcuma caesia* is a perennial monocot herb with fleshy rhizomes. Root stock is large and sessile tubers with pale grey inside [3]. Dried roots and rhizomes are the plant parts of interest of its different properties. They can be used as carminative, antispasmodic, cosmetic, treatment of headaches, rheumatic pains diarrhea [4] etc., cosmetic, externally used for sprains and bruises [5]. It is used for the treatment of liver ailments and jaundice, menstrual problem, chill and cold. It is also used as spice and coloring agent in food. Essential oil is used as analgesic, antipyretic, antimicrobial, antifungal, antihelmintic [6] etc. As long as 4,000 years ago, records from traditional healers of India and China mention its oral use as a remedy for many conditions, including eye infections, intestinal worms, leprosy and different skin diseases [7].
This preliminary study aimed to investigate extracts of *Curcuma caesia* for antibacterial activity in regards to selected gram negative and gram positive bacteria. An attempt was also made to make a phytochemical profile of the plant extracts and establish potential for toxicity using the brine shrimp lethality test (cytotoxic activity).

2. Materials and Methods
2.1. Collection and Extraction of Plant Materials
The fresh plants were collected from Lalitpur District and were duly identified as *Curcuma caesia* in Department of Plant Resources (Banaspati Bibhag), Thapathali, Kathmandu. The plant materials were cut into pieces and were shade dried at room temperature. Dried sample was crushed into powder by electric blender and subjected to extraction by using Soxhlet apparatus. 40 grams of dried and powdered material was extracted separately and successively with 200 ml of petroleum ether, diethyl ether, chloroform and methanol.

2.2. Phytochemical screening
The phytochemical screening was done to identify the main group of chemical constituents present in different extracts of Curcuma caesia by their colour reactions with different

reagents. Each extract was subjected for glycosides, flavonoids, reducing sugars, tannins, and saponin tests using test procedures as mentioned in *Annex 1*.

2.3. Test Organisms

Four pathogenic microbes were used which included two gram positive bacteria; *Staphylococcus aureus* (clinical isolate) and *Enterococcus faecalis* (clinical isolate) and two gram negative bacteria; *Escherichia coli* (clinical isolate) and *Pseudomonas aeruginosa* (clinical isolate). All the microbes were obtained from the Microbiology Laboratory, Department of Microbiology, Tribhuvan University Teaching Hospital and were maintained on Tryptone soya agar slant.

2.4. Media and reagents

The Brine Shrimps eggs were purchased from Aqaculture innovations (Gramhamstown 6140, South Africa). Tryptone soya agar and tryptone soya broth were purchased from Himedia Laboratory pvt Ltd (Mumbai, India). Dimethylsulfoxide (DMSO) was purchased from SIGMA (Poole, Dorset, England), Amoxicillin susceptibility test disc (10 mcg) were purchased from Oxoid (Oxoid Basingstoke, Hampshire, England) and Muller Hinton agar media was purchased from SIMCA (Laboratories suppliers, Kathmandu).

2.5. Screening for antimicrobial activity

Antimicrobial activities were determined by the cup diffusion method. Anitmicrobial screening was performed in the four extracts of the plants; petroleum ether extract, diethyl ether extract, chloroform extract and methanol extract. 16 mg/ml concentration of each plant extract was prepared by dissolving in suitable solvent i.e 50% DMSO (Dimethyl sulphoxide).

The prepared and sterilized Muller Hinton Agar media (*Annex 2*) was poured into sterile petri plates of size 90 mm diameter such that each plate contained 20-25 ml of medium. The plates were allowed to cool for 15-20 minutes and kept in refrigerator for solidification. Bacterial suspension was prepared by inoculating loop full of bacteria in Brain Heart Infusion. Cups were made in agar plates with the help of sterile cork borer of diameter 9 mm and labeled properly. The pure form of bacterial suspension was swabbed on the media with a sterile cotton swab in sterile condition and allowed to dry. To the different cups, 0.1 ml of 16 mg/ml concentration of each extract was placed with the help of micropipette. Standard amoxicillin 10 mcg disc was also placed at the center. All the plates were incubated at 37 ^{0}C for 18-24 hours. The zone of inhibition was measured and compared with standard. The experiments were repeated thrice.

2.6. Brine shrimp bioassay

A method utilizing brine shrimp is proposed as a simple bioassay for natural product research and is considered a useful tool for preliminary assessment of toxicity because they provide a quick and inexpensive alternative to vertebrate testing [8, 9]. The brine shrimp bioassay is based on the ability to kill laboratory cultured brine shrimp (*Artemia salina*) which belongs to phylum arthopoda and class crustaceae [10]. The procedure determines LC_{50} value in mcg/ml of test samples against nauplii in the brine medium [10]. If LC_{50} value of the test sample is less than 1000 mcg/ml, the extract is considered biologically active [9].

*Preparation of test samples***:** 50 mg of crude extract to be tested was dissolved in 5 ml of suitable solvent depending upon its solubility. The methanol extract was dissolved in distilled water while remaining three extracts were dissolved in acetone. This solution was called as stock solution.

Preparation of sea water: Artificial Seawater was prepared by dissolving different chemicals as in stated amount in distilled water as mentioned in *Annex 3*.

Hatching the shrimp: Hatching of brine shrimp was done in the beaker with 300 ml of sea water by sparkling about 50 mg of brine shrimp eggs. This was then illuminated with table lamp of 100 watt for twenty-four hours to achieve the temperature of about 35 °C to hatch nauplii. After 24 hours, the nauplii were collected by dropper.

Application of the test sample and brine shrimp nauplii to the test tubes: From the stock solution 500 µl (equivalent to 1000 ppm), 50 µl (equivalent to 100 ppm) and 5 µl (equivalent to 10 ppm) were transferred to total of fifteen different test tubes, five test tubes for each dose level after evaporating the solvent. Similarly in the other three test tubes, the process was repeated by taking 500 µl, 50 µl & 5 µl of the solvent as control group. The nauplii were counted macroscopically in the stem of the dropper against the lighted background and ten matured and highly motile shrimp larvae were then transferred to each test tube and the volume was made up to 5 ml on each test tube by adding the seawater. Similarly ten matured brine shrimp larvae were transferred in each test tube of control group and the volume was made 5 ml in each.

Counting of nauplii

After 24 hours, the test tubes were observed and the number of survived nauplii in each test tube was counted using magnifying glass. From the data obtained, the LC_{50} value was calculated.

Data Analysis [9]

Cytotoxicity test depends on the calculation of LC_{50} value. The LC_{50} value for the given extract is the lethal concentration that is required to kill the 50% of the brine shrimp nauplli. LC_{50} value can be calculated as follows:

If 'n'= the number of replicates (here three), 'x'=the log of concentration in mcg/ml (here log10, log100, log1000 for three doses levels respectively) and 'y'= probit value (i.e., average survival of all the replicates number of death).

We have,

$$\alpha = \frac{1}{n}[\Sigma y - \beta \Sigma x] \quad \text{---------- (1)}$$

$$\text{Where, } \beta = \frac{\Sigma xy - (\Sigma x \Sigma y)/n}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}} \quad \text{---------- (2)}$$

From probit regression,

$$Y = \alpha + \beta X \quad \text{---------- (3)}$$

$$X = \frac{(Y - \alpha)}{\beta} \quad \text{---------- (4)}$$

Where Y is constant having value 5 for calculating LC_{50}-value. Thus, the LC_{50}-value can be given as,

LC_{50} = Anti log X ---------- (5)

With the help of the above expression, the LC_{50}values for different extracts were determined.

3. Results and Discussion

The extractive preliminary phytochemical analysis performed earlier results the presence of alkaloids, glycosides, flavonoids, tannin etc. It was qualitative analysis only, performed to find out and predict why the plant has anti-microbial and cytotoxic effects. The various groups that were found to be present in the different extracts are listed in Table 1.

Table 1: Phytochemical screening

Extract	Alk	AnthrGly.	Terp	DeoxSug	Tan	Flavo	Sap	Red. Sug	C-Gly.
Petroleum ether extract	*Pre*	Abs	*Pre*	*Pre*	Abs	*Pre*	Abs	Abs	Abs
Diethyl ether extract	*Pre*	Abs	*Pre*	*Pre*	Abs	*Pre*	Abs	*Pre*	*Pre*
Chloroform extract	*Pre*	Abs	*Pre*	*Pre*	Abs	*Pre*	Abs	*Pre*	*Pre*
Methanol extract	*Pre*	Abs	*Pre*	*Pre*	Abs	*Pre*	Abs	*Pre*	*Pre*

The preliminary phytochemical screening of the plant was found to exhibit the positive tests for terpenoid, alkaloid, deoxy sugar and flavonoid; and negative tests for anthraquinone glycoside, tannin and saponin.

Table 2 shows the result of preliminary antimicrobial activity of plant extracts. The results showed that none of the extracts were active against *S. aureus, E. faecalis, E. coli* and *P. aeroginosa*. Control 50 % DMSO had no inhibitory activity against any bacteria.

None of the extracts (petroleum ether extract, diethyl ether extract, chloroform extract and methanol extract) inhibited the gram-positive bacteria (*S. aureus* and *E. fecalis*) and gram-negative bacteria (*E. coli* and *P. aeroginosa*). Since tannin was absent in all the extracts of *C. caesia*, it may be a possible reason for not showing any antibacterial activity [11, 12]. However, detail phytochemical and biological studies are required to confirm these results.

Table 2: Preliminary antimicrobial activity test of extracts of the plant

Extracts	Dose mg/cup	Microorganisms			
		Gram positive		Gram negative	
		S. aureus	*E. faecalis*	*E. coli*	*P. aeroginosa*
Petroleum ether extract	1.6	-	-	-	-
Diethyl ether extract	1.6	-	-	-	-
Chloroform extract	1.6	-	-	-	-
Methanol extract	1.6	-	-	-	-

Note:
(+) Zone of inhibition between 10 - 15 mm
(++) Zone of inhibition between 15 - 20 mm
(+++) Zone of inhibition between 20 - 25 mm
(-) No zone of inhibition

All four extracts of the plant showed cytotoxic activity against brine shrimp nauplii. From Table 3, Brine shrimp bioassay revealed that petroleum ether extract, diethyl ether extract, chloroform extract and methanol extract showed LC_{50} value of 18.923 mcg/ml 1.086 mcg/ml, 45.289 mcg/ml and 100 mcg/ml respectively. The LC_{50} values, thus, indicate the cytotoxic property of the extracts since these values are less than 1000 mcg/ml [33]. Various literatures have shown that *Curcuma longa* possesses antitumour and cytotoxic activity due to the presence of alkaloid curcumine [7, 13].

Table 3: Effects of different extracts on Brine shrimp

LC_{50} (µg/ml)				18.923				1.086				45.289				100		
X				1.277				0.036				1.656				2		
α				0.4				4.93				-1.13				-1.2		
β				3.6				1.9				3.7				3.1		
$\sum x^2$				14				14				14				14		
$\sum xy$				52.8				56.2				45				36.2		
$\sum y$				22.8				26.2				18.8				15		
$\sum x$				6				6				6				6		
x^2	1	4	9		1	4	9		1	4	9		1	4	9			
y	2.8	10	10		6.2±0.33	10±0.33	10± 0		2.6	6.2	10		2.6±0.33	3.6±0.33	8.8±0.81			
logZ (x)	1	2	3		1	2	3		1	2	3		1	2	3			

S.N.	Plant extract	Conc^n (Z)
1	Petroleum ether	10 100 1000
2	Diethyl ether	10 100 1000
3	Chloroform	10 100 1000
4	Methanol	10 100 1000

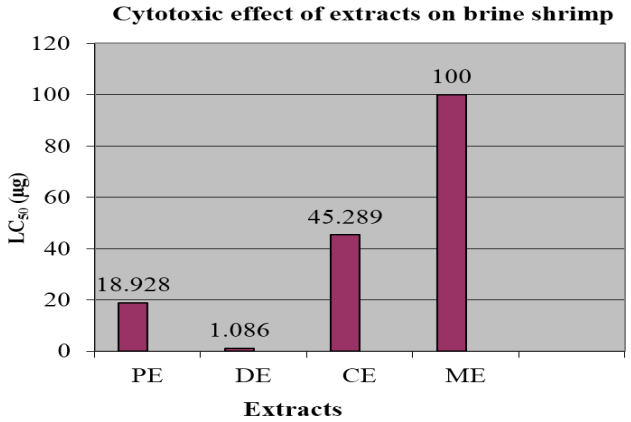

Fig 1: Cytotoxic effect of extracts of the plant on brine shrimp

Annexes

Annex 1: **Test Procedure for Phytochemical Screening**

Test for alkaloids: Dry 6 ml concentrated solution to form residue and dissolve in 1.5 ml 2% HCL, then divide into two parts. To first part add 2-3 drops of Mayer's reagent and to second part add 2-3 drops of Bertrand's reagent separately. A white yellowish precipitate of first tube and white precipitate of second tube indicates presence of basic alkaloids.

Test for Cardiac Glycosides: Test for deoxysugars (Keller-Killiani test): To 2 ml extract, add glacial acetic acid, one drop 5% $FeCl_3$ and conc. H_2SO_4. Reddish brown color appears at junction of the two liquid layers and upper layer appears bluish green.

Modified Borntrager's Test for C-Glycosides: To 5 ml extract, add 5 ml 5% $FeCl_3$ and 5 ml dil. HCl. Heat for 5 minutes in boiling water bath. Cool and add benzene or any organic solvent. Shake well. Separate organic layer, add equal volume dilute ammonia. Ammonical layer shows pinkish red color.

Test for tannin: 0.5 ml extract diluted by 1 ml water and add few drops $FeCl_3$. Blue blackish colour indicates the presence of tannins.

Tests for flavonoid: 4 ml solution + 1.5 ml 50% methanol, warm + metal magnesium + 5-6 drops of conc. HCl. Red (flavonoids), Orange (flavones) and Violet (flavonones).

Test for reducing compounds: 0.5 ml extract diluted by 1 ml water and add 0.5 ml Fehling solution (1+2) and warm. Red brick colour indicates the presence of reducing compounds.

Test for saponins: 2 ml extract place in a test tube and shake for 15 sec. Presence of foam column of 1 cm indicates the presence of saponins.

Test for Terpenoid and Steroid: 4 mg extract + 0.5 ml acetic anhydride + 0.5 ml chloroform and add slowly conc. H_2SO_4.

1. Red violet colour (terpenoids) 2. Green bluish colour (steroids)

Annex 2: **Preparation of Muller Hinton Agar**

Composition of Mueller-Hinton Agar

S. No.	Composition	Amount (gram/litre)
1.	Beef infusion	300.00
2.	Acid Hydrolysis of Casein	17.50
3.	Starch	1.50
4.	Agar	17.00

(Final pH 7.3± 0.1 at 25 °C)

Annex 3: **Preparation of Artificial Seawater**

Composition of artificial sea water

S. No.	Composition	Amount (gm/1000 ml)
1	Sodium Chloride (NaCl)	23.50
2	Potassium Chloride (KCl)	0.68
3	Sodium Bicarbonate ($NaHCO_3$)	0.196
4	Sodium Sulphate (Na_2SO_4)	4.00
5	Boric Acid	0.027
6	Calcium Chloride ($CaCl_2.2H_2O$)	1.78
7	Magnesium Chloride ($MgCl_2.2H_2O$)	10.68
8	Sodium EDTA (Na_2 EDTA)	0.003

4. Conclusion

The above preliminary research showed the different extracts (PE, DE, CE and ME) of *Curcuma caesia* did not exhibit antimicrobial property against the selected gram positive and gram negative bacteria. But this research work found that the plant possessed significant cytotoxic property against brine shrimp nauplii. Further study is needed to search for antimicrobial property and advanced study is needed to confirm the cytotoxic effect of the plant.

5. Conflict of Interest

All authors declare that there is no conflict of interest.

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