Phytochemical and Biological Investigation of *Curcuma amada* Leaves

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ABSTRACT: Methanolic extract of *Curcuma amada* leaves was investigated for isolation of secondary metabolites by successive chromatographic separation (VLC, column chromatography and TLC) which yielded several purified compounds. Among them the structures of β-sitosterol and stigmasterol were determined by analysis of high resolution ^{1}H NMR spectroscopic data and co-TLC with authentic samples. The crude methanolic extract of *C. amada* leaves and its different fractionates i.e. petroleum ether (PESF), chloroform (CSF) and aqueous (AQSF) soluble fractions were evaluated for antibacterial, antioxidant, cytotoxic and thrombolytic activities. In antibacterial screening, the CSF exhibited the highest inhibition against bacterial growth having zone of inhibition 12 mm compared to the standard kanamycin where the zone of inhibition was observed at 18 mm against gram negative *Vibrio mimicus*. Significant free radical scavenging activity was also exhibited by CSF with the IC_{50} value of 103.09 µg/ml as compared to tert-butyl-1-hydroxytoluene (BHT) having IC_{50} 31.88 µg/ml. In cytotoxicity study, the crude extract showed significant lethality towards brine shrimp having with the LC_{50} value of 6.540 µg/ml as compared to the standard vincristine sulfate (0.451µg/ml). In the study for thrombolytic property, different extracts of *C. amada* exhibited clot lysis ranging from 17.24 to 43.55% as compared to standard streptokinase (93.75 %).

Key words: *Curcuma amada*, Sitosterol, Stigmasterol, Antimicrobial, Antioxidant, Thrombolytic activity.

INTRODUCTION

Curcuma amada, also known as mango ginger (Amada), is indigenous to tropical region especially in India, Thailand, China, Malaysia, Indonesia, Northern Australia and Bangladesh.[1] The whole plant is used in indigenous system of medicine as a potential medicinal agent. *Curcuma* (*Cúr-cu-ma*) is a genus of about 80 accepted species in the family Zingiberaceae that contains such species as turmeric and mango-ginger. Mango-ginger is botanically related to neither mango nor ginger. It is a unique spice having morphological resemblance with ginger *(Zingiber officinale)* but imparts a raw mango (*Mangifera indica*) flavor. Various types of chemical constituents have been isolated from mango ginger like curcuminoids, terpenoids, volatile constituents, free phenolic acids etc.[2]

The biological activities of mango ginger include antioxidant, antibacterial, antifungal, anti-inflammatory, platelet aggregation inhibitory, and cytotoxic activities.[3] The objective of the present work was to isolate chemical constituents followed by their biological evaluation.

MATERIALS AND METHODS

Leaves of *C. amada* were collected from the medicinal plant village, Natore, Bangladesh in 2010 and authentication of the plant sample has been confirmed by Mr. Mostafijur Rahman, Assistant Professor, Department of Botany, University of Rajshahi.

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Extraction and isolation. About 800 gm of powdered leaves were taken in a clean flask and methanol was added for cold extraction process. The whole mixture was filtered through cotton and the solvent was evaporated by using a rotary evaporator. The weight of the crude extract was 32.01 gm. About 21.05 gm of extract was fractionated by vacuum liquid chromatographic (VLC) technique using silica gel and dichloromethane, ethyl acetate and finally methanol in increasing order of polarities. Different fractions were collected in beakers according to the TLC analysis designated as 1A, 1B, 1C, 1D, 1E, 1F etc. One of the fractions formed crystals which were then recrystallized to give compound 1 (COMP-1). VLC fraction 1D was subjected to silica gel column chromatography with various solvent systems. Numerous fractions were collected which were denoted as 4A, 4B, 4C, 4D, 4E, 4F, 4G, 4H etc. Then 4E fraction showed crystals which were then recrystallized and washed by methanol. The residue left in the bottom of the test tube was transferred to a vial. By TLC analysis a single band of pink color was found after spraying with conc. sulfuric acid. This compound was termed as compound 2 (COMP-2).

Identification by Nuclear Magnetic Resonance Spectroscopy. The ^{1}H NMR spectra was acquired on a Brucker (400 MHz) instrument in $CDCl_3$ from Bangladesh Council of Scientific and Industrial Research (BCSIR) and Wazed Miah Science Research Centre, Jahangirnagar University.

Biological investigations. The methanolic crude extract (ME) was subjected to solvent-solvent partitioning by using the protocol designed by Kupchan[4] and modified by Van Wagenen *et al.*[5] The crude extract of leaves (10.0 gm) was dissolved in 10% aqueous methanol to make the mother solution which was successively partitioned to give petroleum ether (PESF), chloroform (CSF) and aqueous (AQSF) soluble fractions.

Anti-bacterial activity. The samples were tested for antibacterial activity by the disc diffusion method.[7] Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Kanamycin) discs and blank discs were used as positive and negative control, respectively. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter.[6,7]

Antioxidant activity. The antioxidant potential was determined by using DPPH free radical scavenging assay method and compared to that of tert-butyl-1-hydroxytoluene (BHT).[8] In short, calculated amount of BHT was dissolved in methanol to get a mother solution having a concentration of 1000 µg/ml. Then serial dilution was made to get different concentrations from 500.0 to 0.977 µg/ml. Calculated amount of petroleum ether, chloroform and aqueous fraction of methanolic extract of *C. amada* were dissolved in methanol to get mother solution having a concentration of 1000 µg/ml. Then serial dilution was made to get the concentrations from 500.0 to 0.977 µg/ml.

Then 2.0 ml of the methanol solution of the sample (control/extracts) at different concentrations from 500.0 to 0.977 µg/ml were mixed with 3.0 ml of DPPH methanol solution (20 µg/ml). After 30 minutes of reaction period at room temperature in dark place, the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer.[9] Inhibition of free radical DPPH in percent (I %) was calculated as follows:

$$(I\%) = (1 - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of control reaction (containing all reagents except the test material)

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted by inhibition percentage against extract concentration.

Cytotoxic activity. Cytotoxic activity of the crude extract and its different fractions were determined by brine shrimp lethality bioassay method as described by Meyer *et al.*[10] All the test samples ME, PESF, CSF, AQSF were taken in vials and dissolved in 200 µl of dimethyl sulfoxide (DMSO) to get stock solutions. These solution were used to test the lethal effect against the brine shrimp *Artemia salina* nauplii.

Thrombolytic activity. The *in vitro* thrombolytic activity of the test samples of *C. amada* was determined according to the method reported earlier[11] and percentage of clot lysis was calculated by using the formulae shown below:

% clot lysis = (Weight of the released clot/Weight of clot before lysis) × 100.

RESULTS AND DISCUSSION

Chemical investigations of *C. amada.* A total of eight compounds were obtained from VLC fractions of crude methanolic extract of the leaves of *C. amada*. Among them two of the compounds COMP-1 and COMP-2 were β-sitosterol and stigmasterol. Rest of the compounds could not be characterized due to lack of proper information in NMR spectra of impure compounds

Characterization of COMP-1 as β-sitosterol. β-sitosterol was obtained as white color needle shaped crystals. It showed violet color on the plate after spraying with conc. sulfuric acid regeant followed by heating at 110°C for 5-10 minutes. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of β-sitosterol (Figure 1) was almost identical to that recorded for authentic sitosterol.[12] Co-TLC with a sample of sitosterol previously isolated in our laboratory confirmed its identity as β-Sitosterol.

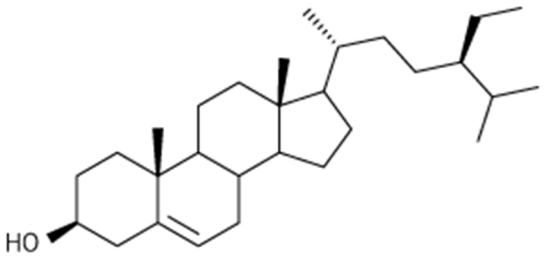

Figure 1. β-Sitosterol

Characterization of COMP-2 as Stigmasterol. The white needle shaped crystals of compound 2 was soluble in chloroform. It produced violet color on TLC after spraying with conc. sulfuric acid followed by heating at 110°C for 5-10 minutes. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of stigmasterol (Figure 2) displayed signals characteristic of a steroidal compound. Comparison of the spectrum with that acquired for authentic sample of stigmasterol as well as co-TLC confirmed the identity of that compound 2 as stigmasterol.

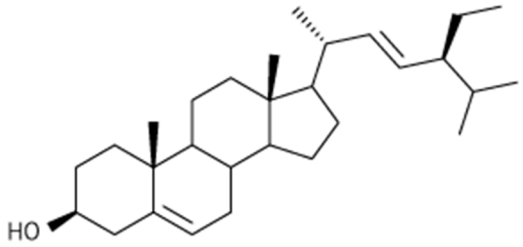

Figure 2. Stigmasterol

Anti-bacterial activity. The chloroform soluble fraction (CSF) exhibited the highest inhibition of bacterial growth having zone of inhibition 7.0 mm to 12.0 mm. The maximum zone of inhibition produced by CSF was 12.0 mm against *S. aureus* (gm ve+) and *V. mimicus* (gm ve-). The results of *in-vitro* microbial screening of leaf of *C. amada* indicated that CSF and MESF possess better antibacterial activity that also support its traditional use. However, AQSF and PESF revealed mild antibacterial activity. These can be further studied to explore potent antibacterial agents. The results are given in table 1.

Anti-oxidant activity. Among all extractives of *C. amada* leaves the chloroform soluble fractionn (CSF) showed moderate anti-oxidant activity with IC_{50} value of 103.09 µg/ml as compared to standard *tert*-butyl-1-hydroxytoluene (IC_{50}=31.88 µg/ml). Other fractions (AQSF, PESF and ME) showed weak free radical scavenging activity. The IC_{50} value of other fractions are shown in table 2.

Cytotoxicity by brine shrimp lethality bioassay. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples. Vincristine sulfate (VS) was used as positive control and the LC_{50} was found as 0.451 µg/ml. The LC_{50} values of ME, CSF, PESF

and AQSF were found to be 6.540 µg/ml, 13.637 µg/ml, 7.134 µg/ml, 16.66 µg/ml respectively. MESF and PESF showed significant lethality whereas CSF, AQSF are showed moderate activity. In the present bioactivity study the crude extracts, and its different fractions showed positive results indicating that the test samples are biologically active. The results are given in table 3.

Table 1. Antibacterial activity of *C. amada.*

Test Bacteria	MESF	PESF	CSF	AQSF	Kanamycin (30 µg/disc)
Gram positive bacteria					
Bacillus cereus	10	7	7	9	26
Bacillus subtilis	8	-	7	-	27
Staphylococcus aureus	-	9	12	8	25
Sarcinalutea	-	-	-	7	27
Gram negative bacteria					
Escherichia coli	10	8	9	8	27
Salmonella typhi	8	-	8	-	28
Shigella dysenteriae	-	9	7	-	27
Vibrio mimicus	-	-	12	7	18
Vibrio parahemolyticus	-	8	9	-	20

ME: Methanol Extract; PESF: Petroleum ether soluble fraction; AQSF: Aqueous soluble fraction; CSF: Chloroform soluble fraction

Table 2. IC_{50} values of crude extract and partitionates of leaves of *C. amada* and BHT.

Sample	IC_{50} (µg /ml)
ME	749.96
CSF	103.09
PESF	558
AQSF	470
BHT (Std)	31.88

ME: Methanol extract; PESF: Petroleum ether soluble fraction; AQSF: Aqueous soluble fraction; CSF: Chloroform soluble fraction; BHT: *tert*-butyl-1-hydroxytoluene

Table 3. Cytotoxic activity of different fractions of *C. amada.*

Test samples	Regression line	R^2	LC_{50} (µg/ml)
VS	y = 30.8x+60.645	0.973	0.451
ME	y = 37.04x + 19.79	0.912	6.540
CSF	y = 39.46x -5.224	0.983	13.637
PESF	y = 38.05x + 17.53	0.929	7.134
AQSF	y = 34.42x -7.945	0.918	16.66

ME: Methanol extract; PESF: Petroleum ether soluble fraction; AQSF: Aqueous soluble fraction; CSF: Chloroform soluble fraction; VS: Vincristine sulfate

Thrombolytic activity. The percentage of weight loss of clot after the application of crude extract of *C. amada* was taken as the indication of thrombolytic activity. The % of clot lysis by crude methanolic extract of *C. amada* was 43.55% as compared to standard streptokinase (LC_{50}=93.75%). The crude methanolic extract of *C. amada* showed significant thrombolytic activity which supports the traditional use of this plant in various diseases. The results are given in table 4.

Table 4. *In vitro* investigation of thrombolytic activity of *C. amada* leaves.

Sample	% of clot lysis leaf part
ME	43.55
CSF	23.96
PESF	21.82
AQSF	17.24
SK	93.75

ME: Methanol extract; PESF: Petroleum ether soluble fraction; AQSF: Aqueous soluble fraction; CSF: Chloroform soluble fraction; SK: Streptokinase

CONCLUSION

Two compounds were isolated by successive chromatographic separation and purification of a crude extract of *C. amada*. Various types of test were done to evaluate the biological activities of the leaves of *C. amada*. The chloroform soluble fraction (CSF) exhibited maximum zone of inhibition of 12.0 mm against *S. aureus* (gm +ve) and *V. mimicus* (gm ve-).

Among all extractives of *C. amada*, moderate free radical scavenging activity was given by CSF (IC_{50}=103.09 µg/ml) as compared to the standard BHT (IC_{50}=31.88 µg/ml). The LC_{50} values of ME was found to be 6.540 µg/ml as compared to standard vincristine sulfate (0.451 µg/ml). The percentage of clot lysis by ME of *C. amada* (leaf) was 43.55% as compared to standard streptokinase (93.75%) which supports the traditional use of this plant as thrombolytic agent.

So, considering the potential bioactivities, this plant can be further studied to find out its unexplored efficacy and to rationalize its medicinal uses.

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