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Phytochemical Analysis and Evaluation of the Whole Plant Crude Extracts of *Curanga amara* juss.

Nilakshi Gohain*, Anindita Talukdar, Minakshi Baishnab and Mohan Chandra Kalita

Department of Biotechnology, Gauhati University, Assam, India

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ABSTRACT

The present research account is an investigation on the phytochemical content, antioxidant property and haemolytic activity of the whole plant crude extract of Curanga amara Juss.Plant extracts were prepared in three different solvents viz. petroleum ether, chloroform and methanol. Phytochemical screening showed the presence of different plant constituents. Alkaloids, phenolic, flavonoids, steroids and reducing sugar were detected in all three solvent extracts. Methanol extract was found to contain highest concentration (268.61±0.04, GAE/g) of phenol, while flavonoid content was highest (1118.67±0.08, QE/g) for the chloroform extract. Free radical scavenging assay against the 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) proved the strong antioxidant property of the plant. A highest of 98.74±0.01 free radical inhibition was achieved with methanol extract and the value was very close to that obtained with ascorbic acid standard (99.4±0.05). Haemolytic activity was tested for the methanol extract against goat blood normal erythrocytes. At 25mg/mL concentration, only 2.4% of haemolysis was observed for the extract, which suggested non-haemolytic nature of the plant compounds the extract contains. The findings of the present study provided a strong scientific background for recognizing this plant as potential medicinal herb used by many local tribes of North-East India.

Keywords: *Curanga amara*, phenol, flavonoids, erythrocytes, haemolysis.

INTRODUCTION

Curanga amara Juss. (Camera, family: Scrophulariaceae)is amedicinal herb locally used by different Indian communities and tribes for curing various diseases. The plant is an important part of herbal practitioners in North-East India. As part of the ongoing research project, a survey was conducted in tribe dominant Baksa (91°35' E, 26°67' E) district of Assam where traditional herbal practitioner of Bodo and Rabha communities have been using this plant for curing diseases like fever and pain. Due to its bitter taste, the herbal healers of the district prescribe the whole plant juice for curing malaria. Similarly, Adi-Minyong tribe of Arunachal Pradesh (eastern most part of Himalaya located in between 26°30' N and 29°28' N and latitude to 91°31' E and 97°30' E longitude) extensively exploit this plant for curing diseases such as dysentery and cough [1]. Mostly, water extract of the whole plant is consumed for the remediation of these ailments. The plant juice has a strong bitter taste that might be attributed to its strong alkaloids contents and probably a reason for its curing property for malaria. Studies on antihypertensive and antimicrobial properties have also been carried out previously [2, 3].The plant is still underexplored in terms of scientific validation of the available traditional knowledge and probable uses for curing some other disease. To strongly correlate the medicinal properties of a plant to its phytochemical profile, it is important to have the baseline information on the phytochemical contents of the plant. Different phytochemicals present in plants attribute to different biological activities. Isolation of plant based phytochemicals and their application as natural drug has been practiced since prehistoric time [4 – 9]. However, there is no report on such study of the plant *C.amara* and thus strongly motivated us for carrying out the present investigation.

The present study encompasses phytochemical screening of the different solvent extracts of the whole plant of *C.amara* along with its antioxidant and haemolytic assays against the free radical 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and red blood cells (RBC) of goatblood respectively. This is anticipated, the studies carried out here will definitely further enhance the scientific scope of the plant *C.amara* in the field of ethno-pharmacological validation.

MATERIALS AND METHODS

Collection of plant material

The authenticated sample of the whole plant *Curanga amara Juss*. was collected during the months of November and December from the Research and Development (*R&D*) Centre of 'The North Eastern Development Finance Corporation Ltd.' (NEDFI)located at Khetri, Kamrup, Assam, India.

Processing of the collected plant

Collected plant samples were gently washed to remove dust particles. Cleaned plant samples were first shade dried at room temperature (25 ± 1^{o}C) for about one month under dust free condition. Dried plants were grinded and sieved 2–3 times to get the finest powder. About 500 gm of plant material were extracted with three different solvent *viz.*petroleum ether, chloroform and methanol in soxhlet apparatus continuously for six hours. All the three extracts *viz.* petroleum ether extract (PEE), chloroform extract (CHE) and methanol extract (ME) were finally concentrated under reduced pressure in a rotary vacuum evaporator. Solvent free extracts were stored at 4^{o}C for future use.

Chemicals

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Sigma (USA). Gallic acid, quercetin and ascorbic acid (vitamin C) were purchased from Himedia Laboratories, Mumbai, India. All other solvents, reagents and chemicals were of analytical grade and purchased from Sisco Research Laboratories (SRL), India.

Spectroscopic measurements

A UV-Vis spectrophotometer (analytic jena, SPECORD® 50 PLUS, Germany) was used for absorbance measurement of different samples in the study.

Screening of phytochemicals

Phytochemical screening of PEE, CHE and ME of *C. amara was* carried outfollowing the standard protocols [10 – 11].

Determination of total phenolic content

The standard procedure of Folin-Ciocalteau (FC) colorimetry was used for determining the total phenolic contents inPEE, CHE and ME [12 – 13]. Gallic acid was used as the standard phenolic acid to set up the calibration curve. The absorbance of total phenolic was measured at λ_{max} = 765 nm using a UV-Vis spectrophotometer. Total phenolic content was expressed as µg/g gallic acid equivalent (GAE) using the equation obtained from the calibration curve y= 2.683x, R^2=0.9599, where y is the absorbance and x is the gallic acid equivalent (µg/g).

Determination of total flavonoids content

A standard method based on the formation of flavonoid-aluminium complex was employed for determining the total flavonoid contents of PEE, CHE and ME [14].Querecetin was used as the standard flavonoid to set up the calibration curve. The absorbance was measured at λ_{max} = 420 nm using a UV-Vis spectrophotometer. Total flavonoid content was expressedas µg/g quercetin equivalent (QE) using the equation obtained from the calibration curve y=0.348x,R^2 =0.977, where y is the absorbance and x is the quercetin equivalent (µg/g).

Antioxidant activity against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH)

Antioxidant acivity of PEE, CHE and ME of *C.amara* was evaluated against DPPH free radical following the method of Sarikurkcu *et al.* [15].Vitamin C was used as standard antioxidant. Different concentrations (1, 5, 10, 25, 50 & 100 µg/mL) of the three extracts were mixed with DPPH free radical solution (prepared in methanol with a final concentration of 0.2 mM).After vortexing, reaction mixtures were allowed to stand for 30 min in dark at room temperature and absorbance of the solutions was measured at λ_{max} = 517 nm using a UV-Vis spectrophotometer. The scavenging activity (%) of the plant extract was calculated from the following equation

$$\textbf{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control} \times 100]$$

Where, $A_{control}$ = Absorbance of DPPH + methanol
A_{sample}= Absorbance of DPPH + methanol + plant extract or Ascorbic acid

Haemolytic activity

Haemolysis test was carried out following a standard protocolwith some modifications [16, 17].Phosphate buffered saline (PBS) and TritonX100 were taken as negative (minimal haemolytic activity) and positive control (maximum haemolytic activity) respectively. To prepare the erythrocytes suspension, 5 mL of goat blood was collected in a sampling vial containing 4% trisodium citrate ($Na_3C_6H_5O_7$) as anticoagulant. Anti-coagulated blood was centrifuged at 1500 rpm for 5 minutes. The blood plasma (supernatant) was discarded and the erythrocytes cell pellet was washed thrice with sterile PBS (pH 7.2) by centrifugation at 1500 rpm for 5 minutes. The cells were re-suspended in normal saline to 0.5%.

For haemolysis assay, 0.5 mL erythrocyte suspension was mixed with 0.5 mL of ME (5 mg and 25 mg/mL in PBS). The mixture was incubated at 37 ◦C for 2 h followed by centrifugation at1500 rpm for 10 min.The free hemoglobin in the supernatant was measured in UV-Vis spectrophotometer at 540 nm. The hemolysis percentage was calculated according to the following formula:

$$\% \text{ Hemolysis} = \frac{A_t - A_n}{A_c - A_n} \times 100$$

Where, A_t = absorbance of ME
A_n = absorbance of PBS
A_c = absorbance of Triton X100

Statistical Analysis

Data are represented as mean±SD of three independent experiments. Correlations were considered significant at $P<0.05$ for different applied parameters.

RESULTS AND DISCUSSION

The different whole plant crude extracts (PEE, CHE & ME) of the *C. amara* evaluated for phytochemical profiling revealed the presence of many important plant constituents. Out of the 12 principle phytochemicals tested for each extract, ME was found to contain 9 different phytochemicals (Table 1). PEE and CHE showed the presence of 6 and 5 different phytochemicals respectively. In common, all the three extracts showed positive for alkaloids, phenolics, flavonoids, steroids and reducing sugar qualitative assays. Antharaquinones, saponin and carbonyl were absent in all three extracts, while cardiac glycosides, terpenoids and tanins were only found in ME. The presence of three important plant secondary metabolites *viz.* alkaloids, phenolics and flavonoids in PEE, CHE and ME was a scientific validation for the long use of *C. amara* as medicinal herbs. It is well known that phenolic compounds are highly effective antioxidants and can scavenge free radicals that cause oxidative damage [18].Flavonoids are strong protectant against lipid peroxidation and also as antioxidant [19]. Most importantly, alkaloids detected in all extracts can be held responsible for the antimalarial, anti-dysenteric and antitussive properties of the plant. As mentioned earlier, this plant is a common choice of many tribes for the remedy of malaria, dysentery and cough [1]. Previous literatures are also in accordance with the role of plant alkaloids for curing these ailments [21 – 22].Thus, the phytochemical screening of the different solvent extracts of the plant *C. amara further* supported the scientific background for selecting this plant as an important medicinal herb by the local peoples of North-East India since long time.

The quantification of the two phytochemicals *viz.* phenolic and flavonoids in PEE, CHE and ME showed a significant variation in their contents (Table 2). CHE exhibited a prominent content (1118.67, QE/g) of flavonoids but moderatecontent of phenolic (110.43, GAE/g). The phenolic concentration was highest for ME (268.61 GAE/g) but with the lowest (132.56, QE/g) flavonoid content.The phenolic content of ME is relatively very high as found in other plant [23]. The high content of phenolic compounds in ME might be responsible for the strong antioxidant activity observed in this study. From the comparative analysis of the phytochemical content in PEE, CHE and ME, it could be suggested that ME could be the best choice for further evaluation of different fractionations by employing chromatographic technique such thin layer chromatography (TLC). With the highest phenolic content and significant flavonoids amount (132.56, QE/g), ME might be the most effective and curative solvent extract for further application in *in-vivo* study.

The antioxidant activity of *C. amara* extracts was determined by measuring the capacity to donate hydrogen or to scavenge free radicals. Change in the original purple color of DPPH into the different shades of yellow color after incubation with PEE, CHE and ME indicated the reducing potentials of the extracts. Both plant extract and standard drug (ascorbic acid) significantly reduced DPPH radicals with the increasing concentrations (Table 3). For all the

three extracts tested, highest inhibition percentage was achieved at their highest applied concentration (100 µg/mL). PEE, CHE and ME exhibited the inhibition percentages of 82.44%, 86.44% & 98.74% respectively. For the standard drug ascorbic acid, 99.4% inhibition was obtained at the same highest concentration. Thus, the scavenging activity of ME was comparable to ascorbic acid. High phenolic content in ME could be attributed to its strong antioxidant property.

Table1.The phytochemical constituents present in different solvent extracts *Curanga amara*

Phytochemical profile	Petroleum ether extract(PEE)	Chloroform extract (CHE)	Methanol extract (ME)
Alkaloids	+ve	+ve	+ve
Cardiac glycosides	-ve	-ve	+ve
Terpenoids	-ve	-ve	+ve
Reducing sugar	+ve	+ve	+ve
Saponin	-ve	-ve	-ve
Tanins	-ve	-ve	+ve
Carbonyl	-ve	-ve	-ve
Phenolics	+ve	+ve	+ve
Flavonoids	+ve	+ve	+ve
Steroids	+ve	+ve	+ve
Courmarin	+ve	-ve	+ve
Antharaquinones	-ve	-ve	-ve

+ve = present, - ve= absent.

Table2: Total phenolic and flavonoids contents in different solvent extracts of *Curanga amara*

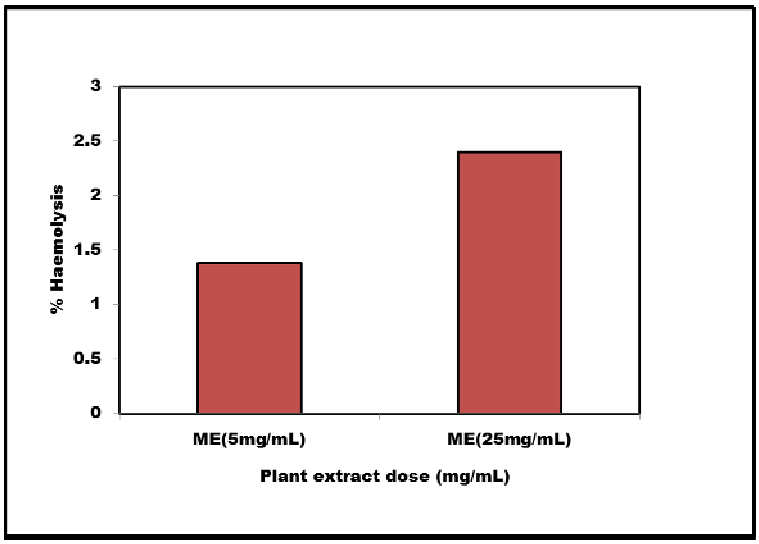
Extract	Total phenol (µg of GAE/g of extract)	Total flavonoids(µg of QE/g of extract)
Petroleum ether extract (PEE)	106.59±0.01	312.64±0.06
Chloroform extract (CHE)	110.43±0.04	1118.67±0.08
Methanol extract (ME)	268.61±0.04	132.56±0.01

Table 3: DPPH scavenging activity exhibited by different solvent extract of *C. amara*

Plant Extract	Applied Concentration ((µg/ml)	Inhibition (%)
Petroleum ether extract (PEE)	1	78.79±0.04
	5	80.46±0.07
	10	80.61±0.01
	25	81.06±0.04
	50	81.08±0.03
	100	82.44±0.02
Chloroform extract (CHE)	1	78.88± 0.01
	5	79.23±0.02
	10	80.43±0.04
	25	80.93±0.07
	50	82.15±0.03
	100	86.44±0.01
Methanol extract (ME)	1	78.75±0.02
	5	80.74±0.06
	10	83.35±0.03
	25	89.71±0.01
	50	97.87±0.02
	100	98.74±0.01
Ascorbic acid	1	80.44±0.04
	5	89.43±0.03
	10	99.2±0.01
	25	99.34±0.02
	50	99.35±0.03
	100	99.4±0.05

Erythrocyte destruction with burst of haemoglobin (haemolysis)is a good manifestation of cytotoxic effect of a test plant material. A negative result of this assay confirms the non-haemolytic nature of the tested material. This assay is important to rule out the possible cytotoxicity of an unknown plant material such as plant extract. Worth mentioning, many plant derived materials show haemolytic activity due to a variety of non-specific mechanisms [24].In the present study, *in-vitro* haemolytic activity was assayed for the ME of *C.amara*. against goat normal erythrocytes. Pertaining to the high phenolic content and highest free radical scavenging activities detected in ME, this particular plant extract was only selected for the *in-vitro* haemolytic activity assay. Two different concentrations (5 & 25 mg/mL) of ME were applied on goat erythrocytes. At 5mg/mLof ME concentration, the haemolysis percentage was only 1.38%, while at 25 mg/mL, it reached upto 2.4% (Figure 1). These degrees of haemolysis are quite negligible and in accord with some previous findings with other plants with much lower plant extract concentrations (maximum of 1mg/mL) [16]. Non-haemolytic nature of ME against mammalian normal erythrocytes

upto 25mg/mL was a significant finding of the present study. High phenolic content, strong antioxidant property and biocompatibility nature against mammalian cell line highly recommended for further research inputs to explore *C. amara* in a more investigative way.



CONCLUSION

The present investigation has evaluated the strong antioxidant and non-haemolytic properties of the underexplored plant *C. amara* juss. Screening of many important phytochemicals, specifically, high contents of phenolic and flavonoids in the crude extracts provided justification to the long time use of this plant in folkloric medicines. This plant has huge potential as source of natural antioxidants that can be extracted out and applied for the remedies of free radical associated human diseases. Importantly, as the plant is not easily available, proper conservation of *C. amara* is strongly suggested.

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