***In Vitro* Anti-Proliferative Activity of *Curcuma angustifolia* and Estimation of Bioactive Compounds for Antioxidant Activity**

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Abstract: The present work deals with preliminary *in-vitro* studies to validate the traditional claims of medicinal properties in consuming *Curcuma* flowers to make the person less susceptible to diseases and the estimation of bioactive compounds responsible for antioxidant activity present. The anti-proliferative activity of aqueous extracts of budding flowers, leaves, full bloomed flowers and mixture of all parts of *Curcuma angustifolia* was studied using MDCK, Vero and HeLa cell lines. The inhibitory activity in all the three cell lines was found to be dose dependent. The extract of leaves and budding flowers exhibited higher inhibitions in MDCK and Vero cells while the full bloomed flowers extract showed higher inhibitory activities in HeLa cells. The bioactive compounds—total phenolics content ranged from 56.96 to 86.77 mg GEA/100 gm which was highest in full bloomed flowers, total flavonoids and the free radical scavenging activity content ranged from 84.74 to 166.11 mg CE/100 g and 43.91 to 64.02 mg AEAC/100 gm respectively, with maximum content in budding flowers. The result showed potential anti-proliferative and antioxidant properties that substantiate the belief of the indigenous people to include the plant in their dietary habits which could be exploited for use in preparation of herbal drugs and supplements.

Key words: *Curcuma angustifolia*, antioxidants, *in-vitro*, phenols, flavonoids, DPPH.

1. Introduction

The indigenous knowledge (IK) refers to the unique, traditional, local knowledge and practices existing within and developed around the specific conditions of indigenous people of a particular geographical area [1] and such traditional knowledge systems are cumulative, representing generations of experiences [2]. *Curcuma angustifolia* belonging to family Zingiberaceae is also commonly known as East Indian arrowroot, which is endemic to India and has been recorded in Central Himalayas, Bihar, West Bengal, Maharashtra, North western and South India [3]. Different parts of the plant are taken by mouth as a dietary aid in gastrointestinal disorders, applied on the skin to soothe painful, irritated or inflamed mucous

membranes [4-6], as weaning food for babies [3] and for various medicinal purposes by the local herbalists [7]. Medicinal plants contain various metabolites and because of these principles they are widely used to cure various ailments. India's use of plants for health care dates back close to 5,000 years and about 8,000 herbal remedies have been codified in the Ayurveda which are still in use [7]. The plants used in traditional medicine are still a large source of natural antioxidant, antimicrobial, anticancer agents that might serve as leads for development of novel drugs [8] and many naturally occurring substances present in the human diet have been identified as potential chemo-preventive agents against cancer [9]. The *Curcuma* plant grows wild in many places of North East India and the local communities of Manipur, Nagaland and Meghalaya consume the early tender

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flowers as food. It is a belief that consumption of flowers during the early stages of seasonal flowering makes the person less susceptible to diseases. The present study deals with preliminary *in vitro* studies to prove the traditional claims of medicinal properties of *Curcuma* flowers. Here, we present the findings of anti-proliferative activity in the aqueous extracts of budding flowers, leaves, full bloomed flowers and mixture of all parts of *Curcuma* plant *in vitro* using Madin Derby Canine Kidney (MDCK), Vero and HeLa cell lines, and the estimation of bioactive compounds responsible for antioxidant activity present.

2. Methodology

2.1 Plant Material

The plant parts of *C. angustifolia* (Fig. 1.) were collected from the wild at a location of 25°68' N latitude and 91°93' E longitude in the state of Meghalaya, India during the flowering season in the months of April to May 2015-16.

2.2 Sample Preparation

The different parts of the plant- budding flowers, leaves, full bloomed flowers and mixture of all parts were triturated in a mortar pestle to make a concentration of 4 g/25 mL with water and ethanol

(80%) respectively. The extracts were homogenized and clarified by centrifugation at 10,000 rpm for 30 min at 4 °C. The aqueous extract was collected and filtered with 0.45 µm syringe filter for *in- vitro* studies. The ethanolic extract was used for estimation of bioactive compounds.

2.3 Anti-Proliferative Assay

Two-fold serial dilution of the aqueous extract was prepared and up to five dilutions (1600, 800, 400, 200, 100 µg/mL) were used for studying the anti-proliferative effects in MDCK, Vero and HeLa cells by 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay (10) with slight modification. The cells @1x10⁶/mL in Earle's Minimum Essential Medium (EMEM, Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS) were suspended in a 96 well plate @100 µL/well. The extracts of various dilutions @100 µL were added in triplicates into each well with non treated control and positive control (Paclitaxel, Sigma Aldrich, USA, @500 µg/mL in two fold dilutions at 50, 25, 12.25, 6.25, 3.12 µg/mL) for HeLa cells. The cells were incubated at 37 °C with 5% CO₂ for 72 hours. Then MTT dye @10 µg was added to each well and further incubated for 4 hours. The supernatants were removed and dimethyl sulfoxide (DMSO) @150

Fig. 1 Plant and flowers of *Curcuma angustifolia* Roxb.

μL was added to each well and incubated at room temperature by gentle shaking for 30 min. The supernatants were collected and the absorbance was measured at 570 nm with an ELISA reader (Lab systems Multiskan Plus, Thermo Fisher Scientific, USA).

2.4 Total Phenols

The total phenolics estimation was done [11] using gallic acid to produce standard calibration curve. To 3.3 mL of distilled water, 0.5 mL sample extract and 0.2 mL of 1N Folin-Ciocalteu's reagent were added and mixed well. After 2 min, 1 mL of 20% Na₂CO₃ was added into it and incubated for 30 min at room temperature. The absorbance was measured at 700 nm and expressed as gallic acid equivalent (mg GAE/100 g).

2.5 Total Flavonoids

Total flavonoids was estimated using the method previously described [12]. Ethanolic extract (1 mL) of the sample was mixed with 0.3 mL of 5% NaNO₂, 0.3 mL of 10% AlCl₃ and 3.4 mL of 4N NaOH at 2 min intervals. The absorbance of the sample was read at 510 nm against reagent blank after 30 min and expressed as catechin equivalent (mg CE/100 g).

2.6 Antioxidant Activity

Free radical scavenging activity in the extract was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as per the method described [13]. To 0.2 mL aliquot of ethanolic extract, 0.3 mL of 100 mM acetate buffer (pH 5.5) and 2.5 mL of 0.1 mM DPPH methanolic solution was added. Change in the absorbance of the sample extract was measured at 517 nm for 30 min and expressed as ascorbic acid equivalent antioxidant capacity (mg AEAC/100g). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percentage inhibition of DPPH by the test sample is calculated by the following formula:

$$\% \text{ Inhibition} = 100 \times (A_0 - A)/A_0$$

where A₀ is the initial absorbance obtained by measuring the same volume of solvent and A is the final absorbance of the sample extract.

2.7 Statistical Analysis

Values of total phenolics, total flavonoids and antioxidant activity were expressed as mean ± standard deviation. Mean comparison was performed using the Tukey's Honest Significant Difference (HSD) test with SAS 9.3 (TS1MO) software package developed by SAS Institute (2000). A difference was considered statistically significant when $p < 0.05$. The data for antiproliferative assay are expressed as mean with their standard errors. Statistical analyses were carried out using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, California, USA) using one-way ANNOVA followed by the Turkey's multiple comparison test. Significance was set at $p < 0.05$.

3. Results

3.1 Anti-Proliferative Activity

The morphological appearance of the cells after 48 hrs of post treatment showed an increase in number of rounded cells when compared with untreated control cells (Fig. 2), and MTT assay showed the aqueous extract of various parts of the plant inhibit cell proliferation in a dose dependent manner. The extract of *C. angustifolia* was found to have inhibitory activity in all the three cell lines when tested at concentrations starting from 1,600 μg/mL in two-fold dilutions up to 100 μg/mL (Fig. 3), whereas up to 400 μg/mL, the inhibitory activity was minimal. At lower concentration up to 100 μg/mL, the MDCK and Vero cells were stimulated when compared with non treated control. The extract of leaves and budding flowers exhibited higher inhibitions in MDCK and Vero cells while the full bloomed flowers extract showed higher inhibitory activities in HeLa cells ($p < 0.05$). The positive control treated HeLa cells showed similar

Fig. 2 Extract of *Curcuma* showing inhibition of cells (*in-vitro*) and formation of formazan crystals in MTT assay.

Fig. 3 Anti-proliferative activity of aqueous extracts of different parts of *Curcuma* plant with a concentration N-16 mg/mL, 1-5- two fold dilution @ 1600, 800, 400, 200, 100 µg/mL. pc- paclitaxel, n @ 500 µg/mL, 1-5 in two fold dilutions @ 50, 52, 12.5, 6.25, 3.12 µg/mL. Values represent the mean ($n = 3$) $\pm$ SD. ($p < 0.05$).

Table 1 Total phenolics, flavonoids and antioxidant activity in different parts of *Curcuma angustifolia* Roxb.

Parts used	Total phenolics (mg GAE/100 g)	Total flavonoids (mg CE/100 g)	DPPH scavenging activity IC50 (mg AEAC/100 g)
Budding flower	56.96 $\pm$ 0.68 ^d	166.11 $\pm$ 0.89 ^a	64.02 $\pm$ 2.18 ^a
Leaves	66.22 $\pm$ 2.95 ^c	112.64 $\pm$ 3.90 ^b	43.91 $\pm$ 0.62 ^b
Full bloomed flowers	86.77 $\pm$ 0.26 ^a	84.74 $\pm$ 1.19 ^c	46.62 $\pm$ 0.34 ^b
Mixture of all parts	76.59 $\pm$ 2.78 ^b	85.24 $\pm$ 2.64 ^c	46.24 $\pm$ 0.44 ^b

Values represent the mean ($n = 3$) $\pm$ SD. Mean followed by different superscripts within column differ significantly ($p < 0.05$).

pattern indicating the inhibitory activity of the extract in dose dependent manner.

3.2 Phenolic Content

Total phenolics content was found to vary significantly ($p < 0.05$) among the different parts and ranged from 56.96 to 86.77 mg GAE/100 g (Table 1) in the increasing order of budding flower < leaves < mixture of all parts < full bloomed flowers.

3.3 Flavonoid Content

Total flavonoids content was also found to vary significantly ($p < 0.05$) and ranged from 84.74 to 166.11 mg CE/100 g (Table 1) in the increasing order of full bloomed flowers < mixture of all parts < leaves < budding flower.

3.4 Antioxidant Activity

The free radical scavenging activity ranged from 43.91 to 64.02 mg AEAC/100 g (Table 1), which was

significantly ($p < 0.05$) higher in budding flowers in comparison to the other three, i.e. full bloomed flowers, leaves and mixture of all parts having similar activity.

4. Discussion

The extract of *C. angustifolia* was found to have inhibitory activity in all the three cell lines in dose dependent manner. Similar inhibitory activities of crude rhizome extract having moderate anti proliferative activity against HeLa cells [14], prostate, colorectal and breast metastatic cell lines [15] have been reported. The extract of leaves and budding flowers exhibited higher inhibitory activity in MDCK and Vero cells while the full bloomed flowers showed higher inhibition in HeLa cells. However, the activity is concentration dependent and up to 400 µg/mL concentration, the inhibitory activity was minimal. Nayak et al. [14] reported the concentration of up to 43.40 µg. mL⁻¹ of rhizome extract has least inhibition

effect on normal cells viability. The anti-cancer agents derived from the edible plants present a big advantage due to their low toxicity. Though most studies used methanolic extracts, we used the aqueous extracts for the study with a view to replicate the natural conditions as possible since the consumption of the parts of plant is either by eating raw or by cooking. It was observed that *C. angustifolia* has cytotoxicity activity causing the death of HeLa cell comparable with the anti-cancer drug (Paclitaxel). Our results also showed that at lower concentration, Vero and MDCK cells are stimulated with significant differences from non treated control indicating the possible immune stimulation. Studies have reported that lining of the intestine of mice after ingestion of curcumin found that CD4+T-helper and B type immune cells were greater in number indicating the localized immune stimulation which will enhance immunity in general [16].

Phenolic compound are commonly available in most of the plants and are reported to be one of most effective antioxidants [17], play an important role in counteracting reactive oxygen species (ROS) thus reducing molecular damage and measurement of total phenolics content could be used to relate their antioxidant properties [18]. Presence of significant amount of phenolics in ethanolic extract of *C. angustifolia* rhizome has been reported [19, 20]. Our result showed the phenolics content vary significantly ($p < 0.05$) among the different parts with highest content in full bloomed flowers. Similar findings have been reported in methanolic extract of *C. angustifolia* rhizome [14].

Flavonoids have been reported to have several health promoting effects viz., anti-inflammatory, antimicrobial activities and effective scavengers of superoxide anions [21, 22]. It is also reported to have strong anticancer activity [23, 24]. Our result showed the total flavonoids content ranged from 84.74 to 166.11 mg CE/100 g with the budding flowers having highest content while Murthy et al. [19] reported the

flavonoid content in ethanolic extract of rhizome to be 41.20 mg quercetine/g. The flavonoids content in leaves of different *Curcuma* species (*C. aeruginosa*, *C. brog*, *C. malabarica*, *C. rakthakanta*, *C. sylvatica*) have been reported to range from 270 to 380 mg ECE (epicatechin equivalents)/100 g (25).

Antioxidants are compounds with free radical scavenging activity and capable of protecting the cells from free radical mediated oxidative stress. Our results of free radical scavenging activity using DPPH assay were higher which corroborates to the findings in ethanolic extract [21], crude extract [26] and methanolic extract [14, 27] of the rhizomes of *C. angustifolia*.

The presence of phenolics, flavonoids and antioxidants in *C. angustifolia* indicates health promoting advantages to the consumers and creating an interest in the natural products having antioxidant properties. Due to its abundance in many parts of India, low cost and safety in consumption, it has been the subject of concentrated scientific research investigating its anticarcinogenic, antibacterial, antifungal, hypoglycemic and antiatherosclerotic activities [14]. Many tribal and rural people in India used this plant in treatments of various diseases [28]. The tribal people used rhizomes in bone fracture, inflammation and intestinal disease [29]. We found the dose dependent activity of the extract from various parts of plant having inhibitory as well as stimulatory effects *in vitro*. The antioxidant activity and metabolite content of various parts of the plant also showed promising results. Further studies on determining the bioactivity and bioavailability of *C. angustifolia* and its active components to investigate its effectiveness against human cancers and other diseases will be desirable so that this plant can be used in preparation of herbal formulations with modern safety standard to meet the health care needs.

5. Conclusion

The present study revealed the various parts of *C.*

angustifolia plant showed potential antioxidant and anticancerous properties as well as stimulatory effects that validates the reason for the indigenous people to include the plant in their dietary habits to remain healthy which could be exploited in preparation of herbal drugs for use in treating various ailments.

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