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Original Research Article

Investigation of antioxidant and cytotoxic activities of methanolic extract from *Curculigo recurvata* leaves

Abstract:

Objective: The present study was designed to investigate antioxidant and cytotoxic effects of the methanol extract of *Circuligo recurvate* leaves, the primary phytochemical property was also intended to screen out.

Method: The antioxidant activity of the extracts were studied using 1,1-diphenyl-2-picryl hydroxyl (DPPH) radical scavenging activity, reducing power capacity assessment and results obtained were compared to standards. The total phenolic contents and flavonoid contents were estimated from Gallic acid and Quercetine calibration curve respectably.

Result: IC_{50} value of the extract showed 879.024µg/ml in case of the DPPH scavenging assay, the total phenolic and the flavonoid contents of extract was 384 mg/gm and 149.23 mg/gm respectively. The reducing power capacity of the extract observed to raise as the concentration of the extract gradually increases. The mortality rate at the assessment of cytotoxicity in brine shrimp napulii model was found increasing with the increase of the concentration and the LC_{50} value of extract was 164.58 µg/ml.

Conclusion: These findings demonstrate that the leaves extract of *C. recurvata* has the presence of antioxidant compound and there is also presence of molecules that can inhibit rapid growing cells.

Keywords: antioxidant, *Curculigo recurvata,* DPPH, methanol extract, phytochemical.

Introduction

Oxidation is essential to many living organisms for the production of energy necessary for biological processes. Oxygen - centered free radicals, also known as reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl (HO), peroxyl (ROO) and alkoxyl (RO) are produced *in vivo* during oxidation. [1] Oxidative stress occurs as a result of an increase in oxidative metabolism, which produces a number of ROS. At a low or moderate concentration, ROS plays in important role in the host defense mechanism against microorganisms. However, by the increase in production of ROS, causes generation of free radicals and oxidants and leads to oxidative stress. Oxidative stress is a deleterious process that can damage cell structures, including lipids, proteins, and DNA, which results onset of a variety of diseases including cancer, inflammation, neurodegeneration, Parkinson's disease, atherosclerosis and pre-mature aging. [2] Virtually all cellular components appear to be sensitive to oxidative damage. Antioxidants are substances known to protect the cells against the effect of free radicals through scavenging free radicals and singlet oxygen. But lipids, proteins, nucleic acids and carbohydrates are all known to undergo oxidative modification. Elimination of synthetic antioxidants in food applications has given more impetus to exploring natural sources of antioxidants. [3]

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The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods or supplements. Ancient times, to improve flavors of food different types of spices were added which were also well known for their antioxidant capacities. Natural antioxidants constitute a broad range of compounds including phenolic compounds, nitrogen compounds and carotenoids.

These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS, and therefore can enhance the immune defense and lower the risk of health complications caused by oxidative stress. [4] In recent years, there is an increasing interest in finding antioxidant activity of the natural products as oxidative stress is found to be responsible for several health complications. Cytotoxicity assays are widely used to screen for cytotoxicity of plant extract either looks for cytotoxic compounds to develop a therapeutic that targets rapidly dividing cancer cells or to screen for unwanted cytotoxic effects before investing in the development as a pharmaceutical product.

Historically, natural products have served as a source for cancer chemotherapy agents. [5] The brine shrimp lethality bioassay (BSLA) has been used routinely in the primary screening of the crude extracts to assess the toxicity towards brine shrimp. The bioassay has a good correlation with cytotoxic activity in some human solid tumors and with pesticidal activity. This *in-vivo* lethality test has been successively employed for providing a frontline screen that can be backed up by more specific and more sophisticated bioassays once the active compounds have been isolated. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay. [6]

C. recurvata belongs to the family of Monocotyledons. It's commonly known as Musali. *C. recurvata* recognized by several leaves, basal, sessile or petiolate; leaf blade usually lanceolate, plicate, leathery or papery. Flowering stems axillary, erect or apically bent downward, long or short. Fruit like a berry, indehiscent, apex sometimes beaked; beak (when present) formed by persistent perianth tube. Seeds are small, often striped and hilum conspicuous. [7] *C. recurvate* is used in the treatment of impotence, limb limpness, arthritis of the lumbar and knee joints, and watery diarrhea in traditional Chinese medicine. It also popular as a potent immunomodulator and aphrodisiac in the Ayurvedic practice. [8]

Materials and Methods

Drugs and Chemicals

DPPH, Methanol and Gallic acid were obtained from Sigma Chemical, USA. Sodium carbonates, Potassium Acetate were obtained from E. Merck, India. Aluminium chloride, Ascorbic acid (AA) and Folin-Ciocalteu reagent were obtained from Sigma-Aldrich, Bangalore, India. All other chemicals in this experiment were of analytical reagent grade.

Plant material and extraction procedures

The plant, *C. recurvate*, was collected from forest of Chittagong Hill Tracts, Bangladesh and authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Bangladesh. After cleaning, the leaves separated and sent for air-

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drying for 10 days. Then kept in an oven at 45°C for 72 hours. About 500gm of the dried powder of leaves were extracted following cold extraction process with sufficient amount of solvent, methanol. Dried powder soaked in methanol for 15 days with occasional shaking and stirring. The final content was filtered by using cotton and Whatmann No.1 filter paper to get the pure extract. Finally, we got the concentrated extract from filtrate by evaporating the solvent in water bath (at 30°C-35°C), the percent yield obtained was 5.4 %.

Preliminary Phytochemical Analysis

Various tests were performed with the methanolic extracts of *C. recurvate* for identification of different phytoconsituents like alkaloids, glycosides, saponinins, tannins, terpinoids, phenolic compounds, protein, and carbohydrates using standard procedures. [9]

In vitro Antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activity of the different *C. recurvata* extracts was determined according to the method by Braca*et al*.2001. [10] 2 ml methanol solution of plant extract or reference standard ascorbic acid at different concentration was mixed with 3 ml methanol solution of DPPH in the test tube. The test tube was incubated at room temperature for 30 min in a dark place to complete the reaction. The absorbance of the solution was measured at 517 nm. DPPH free radical scavenging ability (%) was calculated with the following formula:

$$((A_{\text{absorbance of control}} - A_{\text{absorbance of sample}}) / A_{\text{absorbance of control}}) \times 100$$

The data were presented as mean values ± standard deviation (n = 3).

Reducing Power Activity

The reducing powers of *C. recurvata* extracts were carried out by using the method of Oyaizu M, 1986. [11] Different concentrations of the extracts or standard solutions (1 mL) were mixed with 2.5-mL potassium buffer (0.2 M) and 2.5-mL potassium ferricyanide. After heating for 20 min at 50°C, 2.5-mL trichloroacetic acid (10%) solution was added to the test tube. The total mixture was centrifuged at 3300rpm for 10 min. Next, 2.5-mL supernatant solution was withdrawn from the mixture and mixed with 2.5-mL distilled water and 0.5-mL ferric chloride (0.1%) solution. The absorbance was measured at 700 nm. AA was used for comparison.

Estimation of total phenolic content

Phenolic compounds are a class of antioxidant agents, which was determined by using Shahidi and Wanasundara, 1992. [12] To measure total phenolic content 1ml of plant extract (1000µg/ml) of *C.recurvata* were mixed with 5ml of the Folin-Ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The mixture was shaken thoroughly. Then it was allowed to stand for 20 min at 25^0C. The absorbance of the solution was measured at 760 nm using a spectrophotometer against blank.

Total flavonoid determination

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The total flavonoid content (TFC) was determined using method described by Kumaran and Karunakaran, 2007. In this test, the comparable reference was Quercetin. [10] 1.0 ml of plant extract (1000µg/ml) of *C. recurvate* were added to 3 ml of Methanol and 200µl of 10% aluminium chloride solution, 200µl of 1M potassium acetate solution and 5.6 ml of distilled water was added into the test tube. The test tube was incubated for 30 minutes at room temperature to complete the reaction. Then the absorbance of the solution was measured at 420 nm using a spectrophotometer against blank. TFC was calculated as mg Quercetin equivalent (QE) /100 g sample dry weight.

Brine shrimp lethality bioassay

The cytotoxicity of crude extract was performed at concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL on brine shrimp nauplii using Meyer method. [13] Ten matured shrimps were applied to each of all experimental test tubes and the number of survived nauplii in each test tube was counted after 24 h. From this data, the percentage (%) of mortality of the brine shrimp nauplii was calculated for each concentration using the following formula:

$$\% \text{ Mortality} = {}^{Nt}/_{No} \times 100$$

Where, N_t refers to number of killed nauplii after incubation for 24 h; N_0 is number of total nauplii transferred i.e. 10. The LC_{50} was then determined using regression analysis after plotting % mortality versus log concentration of the drug.

RESULTS

Result of Preliminary Phytochemical Analysis

The extract of *C. recurvate* was tested for different phytoconsituents like alkaloids, glycosides, saponin, tannins, terpinoids, reducing sugars, phenol compounds, protein, carbohydrates and volatile oils using standard procedures and the results are given in Table 1.

SL No:	Tests	Result
1.	Carbohydrate	**+++**
2.	Steroid	**++**
3.	Alkaloid	**+++**
4.	Terpenoid	**-**
5.	Cardiac Glycoside	**+++**
6.	Saponin	**-**
7.	Glycoside	**++**
8.	Tannin	**-**
9.	Resin	**-**

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10.	Protein	-

Table 1: Result of preliminary phytochemical analysis of *C. recurvata* extracts.
(+++) ve= Presence (High), (++) ve = Presence (Medium), (+) ve = Presence (Low) and (-) ve = Absence

DPPH Radical Scavenging Activity:

In case DPPH radical scavenging activity of methanol extract of *C. recurvata* shown in Figure 1 Where increasing the concentration of the extract, activity was found to increase remarkably and the inhibitory capacity of the plant extract was comparatively lower than the ascorbic acid.

Furthermore the methanol extract of *C. recurvata* showed moderate to good DPPH scavenging activity in which IC_{50} value for the plant extracts was 259.040µg mL. In this test, Ascorbic acid was used as the reference antioxidant and the IC_{50} value for ascorbic acid was 27.02µg mL.

Total Phenol and Flavonoid Content:

The total phenol and total flavonoid contents of *C. recurvata* of methanol extract were expressed in gallic acid and quercetin equivalents respectively that are shown in Figure 2 The content of phenolics of the extract under this investigation showed moderate result 384.66 $\pm$ 3.34mg/g GAE and the amount of flavonoid was 149.23 $\pm$ 1.12mg/g quercetin equivalent.

Different studies suggest that different types of polyphenolic compounds such as flavonoids, phenolic acids which are found in plants have multiple biological effects, including antioxidant activity. [14]

Reducing Power:

The plant extracts was identified by using the potassium ferricyanide reduction method. The reductive capabilities of this extract were compared with ascorbic acid, which demonstrated at figure 3 The reducing power of the extract was moderately strong while increasing dose it shows steady state condition.

Brine Shrimp Lethality Bioassay:

The Methanol extract of *C. recurvata* was tested for Brine shrimp lethality bioassay by using brine shrimp nauplii and DMSO as a solvent. Control was used to see whether DMSO had any effect on brine shrimp lethality or not. The control group of brine shrimp nauplii with and without DMSO exhibited no mortality. For the extract, the number of nauplii died and percent mortality was counted. The result is shown in the Figure 4 and Table 2

Concentration(µg/ml)	No of Nauplii	Living	Death	% of mortality	Average	$LogC_{50}$
31.25	10	6	4	40		
62.5	10	5	5	50		

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125	10	4	6	60	78.33	164.583
250	10	2	8	80		
500	10	2	8	80		
1000	10	0	10	100		

Crude extract resulting in LC50 value of less than 1 µg/ml are considered as significantly active which suggest that the *C. recurvata* crude extract, with LC50 value of 164.58 µg/ml has a very low toxicity.

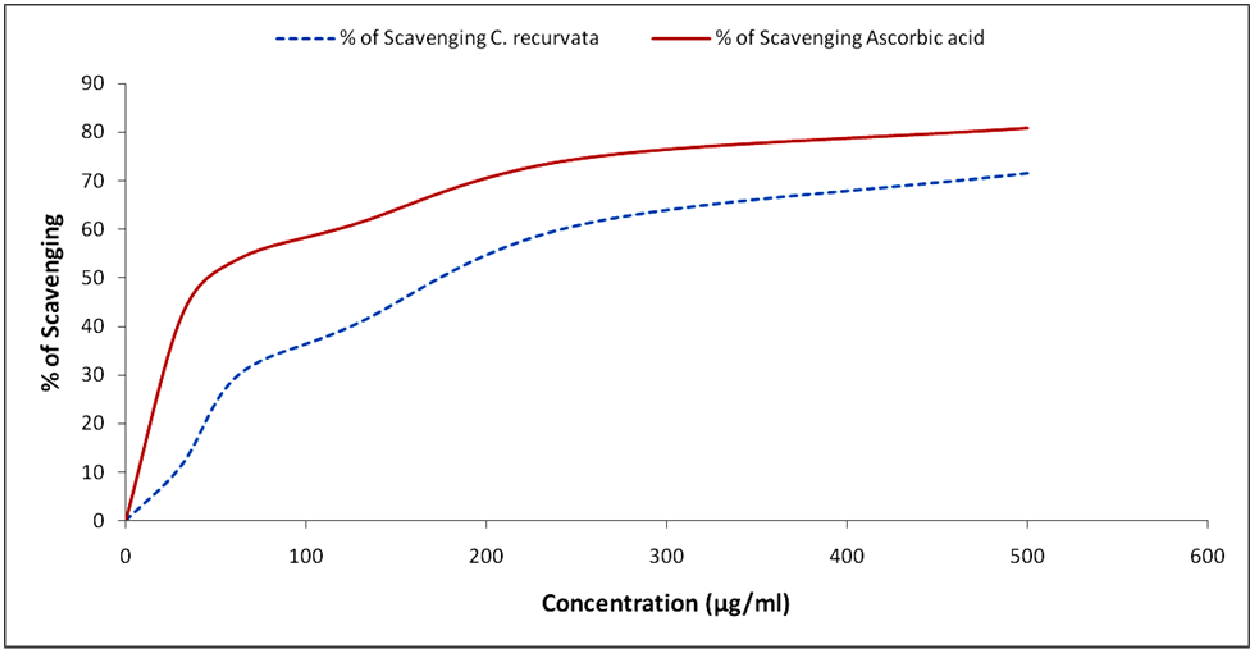

Figure 1: Percentage of DPPH radical scavenging activity of *C. recurvata* with standard Ascorbic acid.

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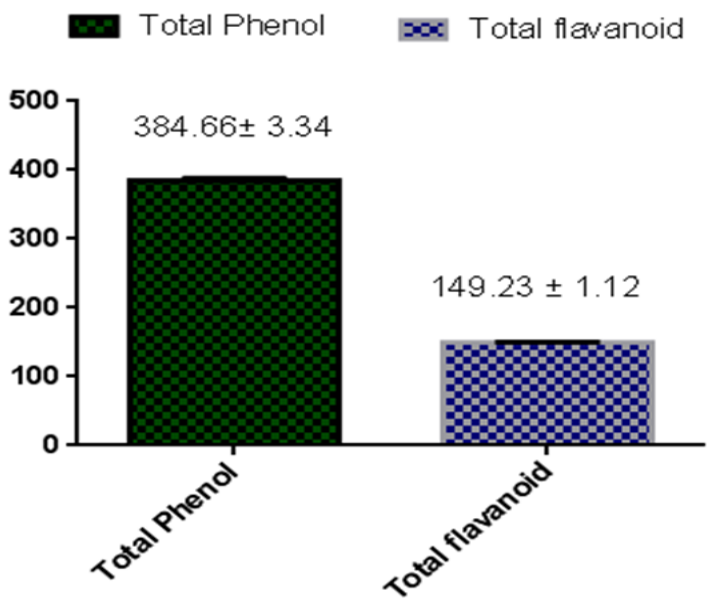

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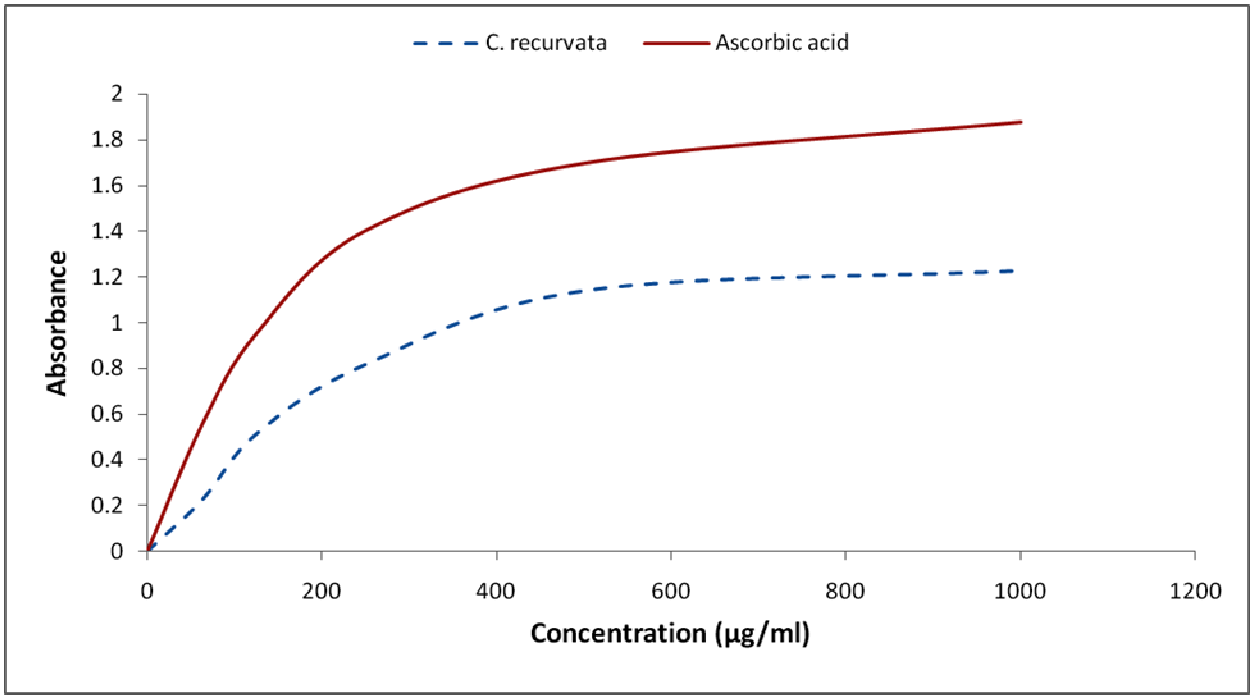
174 Figure 2: Total phenol and Total flavonoid content in *C. recurvata*.



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176 Figure 3: Reducing power of *C. recurvata* with standard Ascorbic acid.

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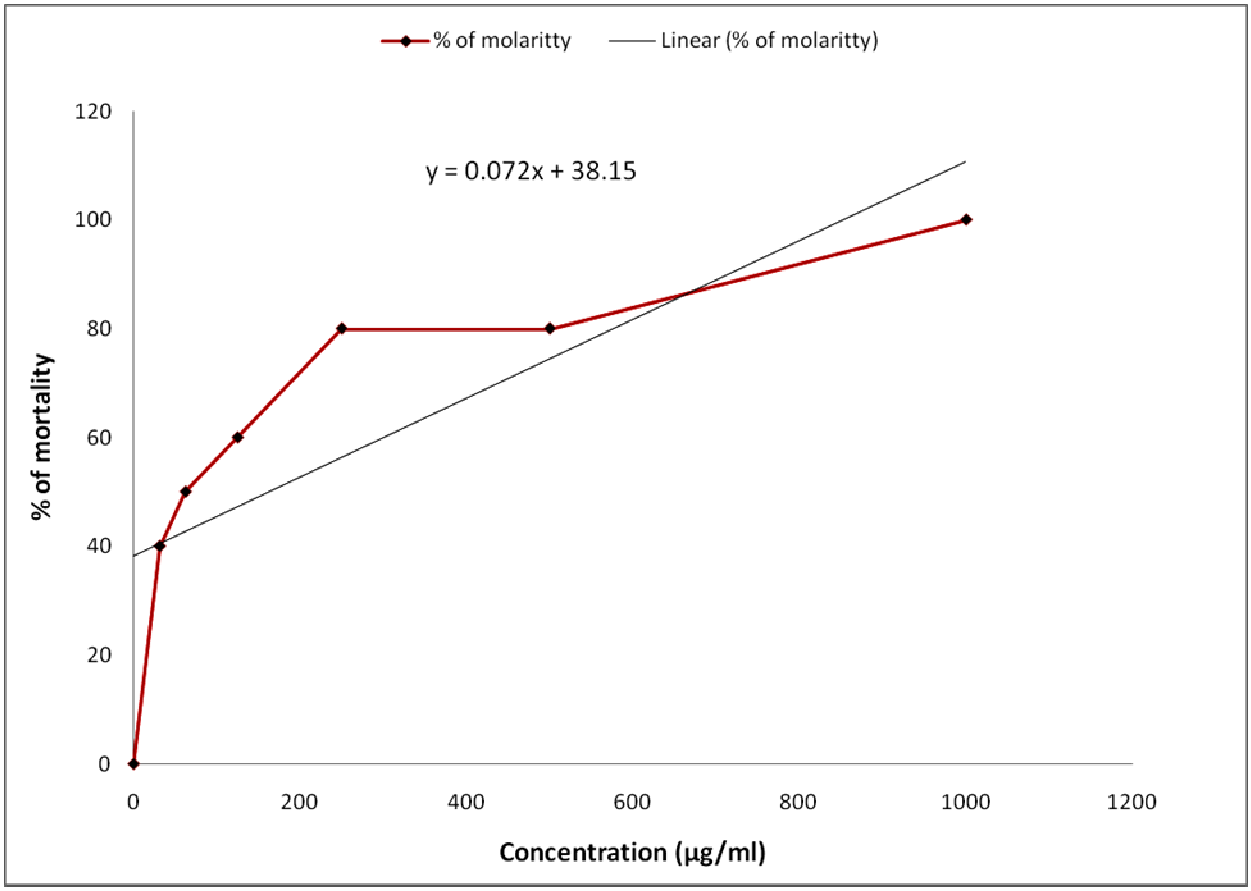

Figure 4: Cytotoxicity of *C. recurvata* extract

Discussion

A lot of modern medicines have been discovered as a result of the scientific follow-up of the traditional herbal preparations. However, now a day, most of the medicines, cosmetics, chemicals etc. are synthetically prepared, however, in many cases their origin is natural more specifically of plants sources.

In addition, a high intake of vitamin C appears to protect against gastric cancer, probably through scavenging ROS formed in the gastric mucosa. Further investigations on vit-C have proved its preventive effects on inhibition of tumor promotion. [15] Flavonoid are polyphenols and they are abundantly found the fruits, vegetables, grains, bark, roots, stems, flowers and tea. [16] They possess anti-inflammatory, anti-allergic, antiviral and anti-carcinogenic properties due to their antioxidant potential. [17]

This was only a preliminary study but the plant need further screening against various disease condition, occurred due to antioxidative component. This will help to find out its unexplored efficacy and can be a potential source of biologically important drug candidates. A number of scientific reports indicate certain terpenoids, steroids and phenolic compounds such as tannins, coumarins and flavonoids have a chemo preventive role in cancer, through their effects on signal transduction in cell proliferation and angiogenesis. [18] The presence of different phytochemicals

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in the studied methanolic extract is the best reason for its prominent antioxidant property. From the quantification of flavonoid content proved that this plant extract has scientific basis in the wide use in Ayurvedic and folk medicine.

The *in vitro* cytotoxicity activities of *C. recurvata* are probably due to the presence of tannin, terpenoids or steroids. Furthermore, flavonoids such as quercetin, kaemferol and their glycosides have been shown to possess antimutagenic and antiproliferative effect in various cancer cell line 21. [19] So, it is suggested that for specification of the mechanism of its action against brine shrimp, next study should be carried out on human cell line or on any other rodent.

Conclusion

The methanolic leave extract of *C. recurvate* is potent against rapid proliferating cell like brine shrimp; it is due to containing various antioxidative molecules. From this present work, we got a promising molecule that could be an antioxidant supplement and will help in the prevention of different oxidation induced malfunction, for sure.

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