



Received on 01 June, 2010; received in revised form 18 August, 2010; accepted 13 September, 2010

## **IN VITRO ANTIOXIDANT AND CYTOTOXIC POTENTIAL OF *COSTUS SPECIOSUS* (KOEN.) SMITH RHIZOME**

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### **ABSTRACT**

#### **Keywords:**

*Costus speciosus*,  
Free radical scavenging,  
*In vitro* antioxidant,  
Cytotoxicity

This study evaluated the *in vitro* antioxidant and cytotoxic potential of methanolic extract of the rhizome of *Costus speciosus* (*Zingiberaceae*). The different antioxidants assays including total antioxidant activity, DPPH, nitric oxide (NO) scavenging, total phenolic content and flavonoid content were studied. *Costus speciosus* exhibited potent total antioxidant activity which was expressed as an equivalent of ascorbic acid mg/g plant extract. In addition, *Costus speciosus* had effective free radical scavenging, nitric oxide scavenging activity, total phenolic content and flavonoid content depending on concentration. These various antioxidant activities were compared with the standard antioxidants ascorbic acid and quercetin. The extract of the rhizome of *Costus speciosus* also demonstrated a strong cytotoxic activity against brine shrimp nauplii with an LC<sub>50</sub> value of 31.55 µg/ml.

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**INTRODUCTION:** Reactive oxygen species are involved in a number of degenerative diseases such as arteriosclerosis, cancer, cirrhosis and diabetes <sup>1, 2, 3, 4</sup> and also in wound healing <sup>5</sup>. Plant-derived antioxidants such as tannins, lignans, stilbenes, coumarins, quinones, xanthenes, phenolic acids, flavones, flavonols, catechins, anthocyanins and proanthocyanins could delay or provide protection for living organisms from damage caused by uncontrolled production of reactive oxygen species (ROS) and the concomitant lipid peroxidation, protein damage, and DNA strand breaking <sup>6</sup> because of their redox properties, which allow them to act as hydrogen donors, reducing agents, free radical scavengers <sup>7, 8</sup>. They are also strong chelators of metal ions <sup>9</sup>. Current interest is focused on the potential role of antioxidants and antioxidant enzymes in the treatment and prevention of arteriosclerosis, heart failure, neurodegenerative disorders, aging, cancer, diabetes mellitus, and several other diseases <sup>10</sup>.

*Costus speciosus* (Koen.) Smith belongs to the family Costaceae, widely distributed in Bangladesh, India and some other tropical countries. Diosgenin is the principle constituent of its rhizome and widely used as starting material in the commercial production of steroidal hormones. The rhizomes are useful in vitiated conditions of kapha and pitta, burning sensation, flatulence, constipation, helminthiasis, leprosy, skin diseases, fever, hiccup, asthma, bronchitis, inflammation and anemia. It is used to make sexual hormones and contraceptives. Alkaloids of *Costus speciosus* have been shown anticholinesterase activity in both *in vitro* and *in vivo* study <sup>11</sup>. Peroral administration of *Costus speciosus* aqueous extract have significant hypoglycemic effects with a simultaneous glucose load <sup>12</sup>. Literature reviews indicated that no studies combining the antioxidant and cytotoxic activities of the rhizome have so far been

undertaken. Taking this in view and as part of our ongoing search on Bangladeshi medicinal plants <sup>13</sup> the present study aimed at evaluating the antioxidant, and cytotoxic potential of the methanolic extract of *Costus speciosus* rhizome.

#### **MATERIALS AND METHODS:**

**Chemicals:** Ammonium molybdate, Folin-chiocaltu phenol reagent, sodium nitroprusside, were purchased from E. Merck (Germany). 1, 1-diphenyl- 2- picryl- hydrazyl (DPPH), sodium nitroprusside, ascorbic acid, quercetin, and potassium ferric cyanide were purchased from Sigma Chemical Co. Ltd, (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

**Plant material:** The rhizome of the plant of *Costus speciosus* was collected from the botanical garden of Pharmacy department of Jahangirnagar University, Bangladesh during January 2008. The plant material was taxonomically identified by the National herbarium of Bangladesh. A voucher specimen no. 33165 is maintained in our laboratory for future reference.

**Preparation of plant extract:** The plant material was shade-dried with occasional shifting and then powdered with a mechanical grinder, passing through sieve #40, and stored in a tight container. The dried powder material (650 g) was extracted with methanol for 72 h in a Soxhlet apparatus. The solvent was distilled under reduced pressure, and the resulting semisolid mass was vacuum dried using rotary flash evaporator to yield a solid residue (18.42% w/w). The preliminary phytochemical analysis was performed to identify the phytoconstituents present in the extract <sup>14</sup>.

**Determination of total antioxidant capacity:** The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method <sup>15</sup>. The

assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH 0.3 ml extract was combined with 3 ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (Shimadzu, UV-150-02) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid using the following formula:

$$C = (c \times V)/m$$

Where: C- total antioxidant activity, mg/g plant extract, in Ascorbic acid; c-the concentration of ascorbic acid established from the calibration curve, mg/ml; V- the volume of extract, ml; m- the weight of pure plant methanolic extract, g.

**Free radical scavenging activity measured by 1, 1-diphenyl-2-picryl-hydrazyl:** The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined by the method<sup>16</sup> Plant extract (0.1 ml) was added to 3ml of a 0.004% MeOH solution of DPPH. Absorbance at 517nm was determined after 30 min, and the percentage inhibition activity was calculated from;

$$[(A_0 - A_1)/A_0] \times 100,$$

Where  $A_0$  is the absorbance of the control, and  $A_1$  is the absorbance of the extract/ standard.  $IC_{50}$  was calculated from equation of line obtained by plotting a graph of concentration versus % inhibition.

**Nitric oxide radical scavenging assay:** The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric

oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered solution (pH 7.4) was mixed with different concentrations of methanol extract of *Costus speciosus* dissolved in methanol and incubated at room temperature for 150 min.

The same reaction mixture without the methanol extract but the equivalent amount of methanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2%  $H_3PO_4$  and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm.<sup>17</sup> The antioxidant activity of the extracts was expressed as  $IC_{50}$ . As like DPPH method  $IC_{50}$  was calculated from equation of line obtained by plotting a graph of concentration ( $\mu\text{g/ml}$ ) versus % inhibition.

**The amount of phenolic compounds and flavonoids:** The total phenolic content of plant extracts was determined using Folin–Ciocalteu reagent<sup>18</sup>. *Costus speciosus* plant extracts (100  $\mu\text{l}$ ) were mixed with 500  $\mu\text{l}$  of the Folin–Ciocalteu reagent and 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined. These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of gallic acid (GAE). Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = (c \times V)/m'$$

Where: C- total content of phenolic compounds, mg/g plant extract, in GAE; c- the concentration of gallic acid established from the calibration curve, mg/ml; V- the volume of extract, ml; m- the weight of pure plant methanolic extract, g.

The flavonoids content was determined by aluminium chloride colorimetric method<sup>19</sup> was modified from the procedure reported by Woisky and Salatino. Quercetin was used to make the calibration curve. The different concentration of plant extract (0.5 ml) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. All determinations were carried out in duplicates. These data were used to estimate the flavonoid contents using a standard curve obtained from various concentration of quercetin. The content of flavonols, in quercetin equivalents was calculated by the following formula:

$$C = (c \times V)/m$$

Where: C- total content of phenolic compounds, mg/g plant extract, in GAE; c- the concentration of gallic acid established from the calibration curve, mg/ml; V- the volume of extract, ml; m- the weight of pure plant methanolic extract, g.

**Brine Shrimp Lethality Bioassay:** The cytotoxic activity of the plant was evaluated using Brine Shrimp lethality bioassay method<sup>20</sup> (Mayer *et al.*, 1982) where 6 graded doses (*viz.*, 5µg/ml, 10µg/ml, 20µg/ml, 50µg/ml, 100µg/ml, 200µg/ml) were used. Brine shrimps (*Artemia salina* Leach) nauplii Ocean 90, USA were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 hours.

The nature nauplii were then used in the experiment. DMSO was used as a solvent and also as a negative control. The median lethal concentration LC<sub>50</sub> of the test sample after 24 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. Vincristine sulfate was used as a reference standard in this case.

**Statistical analysis:** The data is expressed as a mean ± SE of three experiments.

**RESULTS AND DISCUSSION:** The qualitative chemical analysis of the rhizome of *Costus speciosus* showed the positive result for the presence of glycosides, flavonoids, triterpenoids, tannins, and steroids. Antioxidant methods have been proposed to evaluate antioxidant characteristics and to explain how antioxidants function.

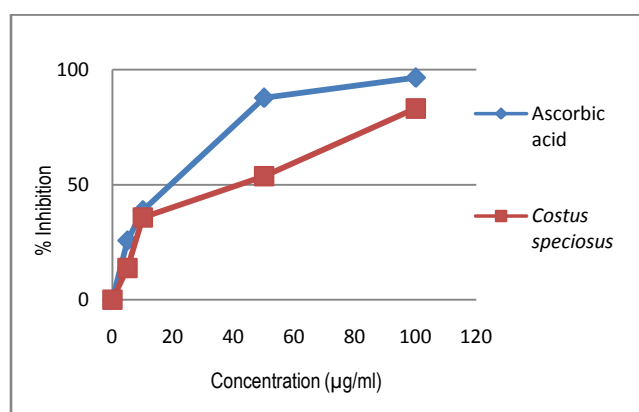
**Effect on total antioxidant activity:** The antioxidant activity due to the presence of phytoconstituents such as flavonoids and biflavones has been reported<sup>21</sup>. The yield of the methanol extract of *Costus speciosus* rhizome and its total antioxidant capacity are expressed as the number of equivalents of ascorbic acid (**Table 1**). The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm.

The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts<sup>15</sup>. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract.

**TABLE 1: YIELD, TOTAL AMOUNT OF PLANT PHENOLIC COMPOUNDS, FLAVONOIDS AND TOTAL ANTIOXIDANT CAPACITY OF METHANOLIC EXTRACT OF *COSTUS SPECIOSUS* RHIZOME**

Yield (%)	Total phenols mg/g plant extract (in GAE)	Total flavonoids mg/g plant extract (in Quercetin)	Total antioxidant capacity equivalent to ascorbic acid mg/g plant extract
18.42%	93.95	9.32	82.88

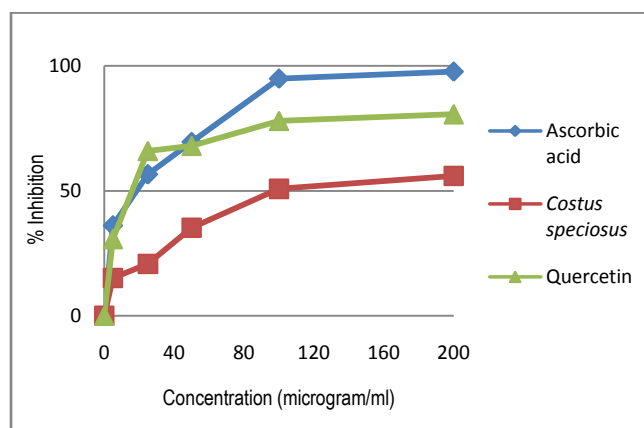
**Effect on scavenging of DPPH radical:** DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule<sup>22</sup>. The absorption maximum of a stable DPPH radical in methanol was at 517 nm. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, results in the scavenging of the radical by hydrogen donation. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity of antioxidants<sup>23</sup>. **Figure 1** illustrates percent (%) of inhibition with increasing the concentration of methanolic extract of *Costus speciosus* rhizome and standard (Ascorbic acid). IC<sub>50</sub> values of *Costus speciosus* rhizome and ascorbic acid were 50.38 and 33.32  $\mu\text{g/ml}$ , respectively.



**FIG. 1: COMPARATIVE % INHIBITION OF DPPH SHOWN BY STANDARD ANTIOXIDANT (ASCORBIC ACID) AND *COSTUS SPECIOSUS* RHIZOME EXTRACT**

### Effect on nitric oxide (NO $\cdot$ ) scavenging activity:

Nitric oxide and superoxide anion cause ischemic renal injury separately, and these radicals work together to bring about further damage. The toxicity and damage caused by NO $\cdot$  and O $\cdot^-$  is multiplied as they react to produce reactive peroxynitrite (ONOO $^-$ ), which leads to serious toxic reactions with biomolecules such as protein, lipids, and nucleic acids<sup>24</sup>. Suppression of NO $\cdot$  released may be partially attributed to direct NO $\cdot$  scavenging, as all concentrations of *Costus speciosus* rhizome decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. A nitric oxide radical generated from sodium nitroprusside at physiologic pH was found to be inhibited by *Costus speciosus* rhizome. Figure 2 illustrates the percentage inhibition of nitric oxide production and it is observed that scavenging of nitric oxide by the extract is concentration dependent. Ascorbic acid and quercetin were used as a reference compound. The concentration of *Costus speciosus* rhizome necessary for 50% inhibition was found to be 97.74  $\mu\text{g/ml}$ . However, 18.63  $\mu\text{g/ml}$ , and 15.97  $\mu\text{g/ml}$  was required for ascorbic acid and quercetin respectively. The extract showed significant level of inhibition of nitric oxide production by activated peritoneal macrophages with *in vitro* conditions.



**FIG. 2: COMPARATIVE % INHIBITION OF NO PRODUCTION SHOWED BY STANDARD ANTIOXIDANT (ASCORBIC ACID AND QUERCETIN) AND *COSTUS SPECIOSUS* RHIZOME EXTRACT**

**Effect of total phenolic compounds:** Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl groups.<sup>25</sup> A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical scavengers.<sup>9</sup> In addition, it has been reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation<sup>26</sup>.

Flavonoids, the most important and most diverse natural phenolics<sup>27</sup> have diverse chemical and biological activities including radical scavenging properties. Therefore, the content of both groups are determined in the extracts (Table 1). The content of total phenolics in the extracts of *Costus speciosus* rhizome is determined using the Folin–Ciocalteu assay, calculated from regression equation of

calibration curve ( $y = 0.013x + 0.127$ ,  $r^2 = 0.988$ ) and is expressed as gallic acid equivalents (GAE) and the flavonoid contents of the rhizome extract in terms of quercetin equivalent (the standard curve equation:  $y = 0.009x - 0.036$ ,  $r^2 = 0.979$ ).

**Assay for cytotoxicity of *Costus speciosus* rhizome extract:** A general Brine shrimp lethality assay, used to measure the cytotoxicity of plant extract and also an indicative of cytotoxicity, antibacterial activities, pesticidal effects and various pharmacologic actions. In cytotoxicity, the extract was highly effective and showed an LC<sub>50</sub> value of 31.55 µg/ml whereas 0.25 µg/ml was observed for vincristine sulfate. The results of cytotoxic activity are presented in **table 2**. An extract having LC<sub>50</sub> below 30 µg/ml is generally considered as a potent bioactive extract. It also indicates that the plant extract, perhaps, possess the potentials to kill cancer cells as well as to kill pests<sup>28</sup>. These activities may lead to conclude the presence of secondary metabolites responsible for showing such biological effects.

**TABLE 2. CYTOTOXICITY OF *COSTUS SPECIOSUS* RHIZOME ON BRINE SHRIMP NAUPLII**

Test sample	Conc. of sample(µg/ml)	Log C	% Mortality	LC <sub>50</sub> test sample (µg/ml)	LC <sub>50</sub> of Vincristine Sulfate (µg/ml)
<i>Costus speciosus</i> (Methanolic rhizome extract)	200	2.303	80	31.55	0.25
	100	2.004	70		
	50	1.698	60		
	20	1.301	40		
	10	1	30		
	5	0.698	10		

**CONCLUSION:** In summary, this study of *Costus speciosus* rhizome extract reveals some interesting activities like cytotoxic and antioxidant activities of this plant. The rhizome extracts have got significant cytotoxic activity

indicate that the plant can be selected for further cell line assay. Moreover, it is clear that *Costus speciosus* rhizome has powerful *in vitro* antioxidant capacity against various antioxidant systems. From the results, it can be concluded

that the antioxidant activity of *Costus speciosus* rhizome was concentration dependent and may be due to the presence of Phytoconstituents such as flavonoids and phenolic compounds in the methanolic extract of *Costus speciosus* rhizome.

**ACKNOWLEDGEMENT:** The authors would like to thank the Department of Pharmacy, Jahangirnagar University for providing plants and lab facilities and also to the Department of Pharmacy, Bangladesh University for providing lab facilities for the research work.

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