

Antioxidant activity of Curculigoside, a phenolic glucoside from *Curculigo orchioides* Gaertn.

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Abstract

Background: Curculigoside is a phenolic glucoside derived from the plant *Curculigo orchioides* Gaertn, a well known immune stimulatory and rejuvenating tuberous plant. A systematic analysis of the antioxidant potential of curculigoside is not reported as far.

Methods: In the present study antioxidant activity of curculigoside was evaluated both in the *in vivo* and *in vitro* models. Tube test were performed to analyse 1, 1-diphenyl-2-picrylhydrazyl (DPPH), super oxide (Nitro blue tetrazolium reduction) and hydroxyl radical (Fenton reaction) scavenging effects. Besides, effect of the compound on lipid peroxidation and nitric oxide scavenging was also tested. *In vivo* antioxidant activity was checked by PMA (Phorbol-12-myristate-13-acetate) induced superoxide radical formation by mouse peritoneal macrophages.

Result: Curculigoside was found to scavenge the hydroxyl radicals generated by Fenton reaction and also inhibited the *in vitro* lipid peroxidation. The compound was also found to be an effective scavenger of superoxide, DPPH and nitric oxide (NO) radicals. Curculigoside showed a significant scavenging effect on the NO release by cultured macrophages. Besides, oral administration of curculigoside inhibited superoxide generation in macrophages *in vivo*.

Conclusion: Curculigoside was found to exhibit good free radical scavenging effects which is comparable to known standards, both in the *in vitro* as well as *in vivo* systems.

Keywords: Curculigoside, free radical scavenging, lipid peroxidation, superoxide dismutase, Nitric oxide.

1. Introduction

Free radicals are highly reactive substances, generated during a number of biochemical reactions in the body, and play an important role in biological processes [1]. At the same time they can act as a root cause of many diseases, including cancer and heart diseases [2,3] when formed in huge amount, or if the natural defences are burdened. Oxidative stress in the body leads to the generation of reactive oxygen species, such as superoxide (O₂), hydroxyl (OH.) and peroxy (.OOH, ROO.) radicals [4]. Even if the body itself possess an antioxidant mechanism, many times it become insufficient to completely remove these radicals to maintain a balance [3,5]. Traditional medicine forms a large source of natural antioxidants that might serve as leads for the development of novel drugs [4].

Phenolic content in plants have been reported to have multiple biological effects, including antioxidant activity [6]. Curculigoside is a phenolic glucoside present in *Curculigo orchioides* Gaertn. and the compound was isolated and characterized Kubo *et al*, 1983 [7]. The compound reported to attenuates human umbilical vein endothelial cell injury induced by H₂O₂ by Wang *et al* [8]. In the present study we have performed a systematic investigation on the antioxidant potential of curculigoside *in vitro* as well as *in vivo* systems.

2. Materials and Methods

1.1 Animals

Balb/c mice (4-6 weeks old, male) were purchased from small animals breeding station, Mannuthy, Kerala, India. The animals were fed normal mouse chow (Krish Scientific Stores, Bangalore, India) and water *ad libitum*. Prior approval of the Institutional Animal Ethics Committee (IAEC) was procured and animal experiments were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Government of India.

1.2 Chemicals and reagents

PMA, DPPH and RPMI 1640 medium were purchased from Sigma-Aldrich, USA. Nitro blue tetrazolium (NBT), 5-5'-dithiobis (2-nitro benzoic acid) (DTNB), were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai. Foetal bovine serum (FBS) was obtained from Biological Industries, Kibbutz Beit Haemek, Israel. All other chemicals and reagents used were of analytical grade.

2.3 Curculigoside

Curculigoside was obtained from Shanghai Standard Biotech, Shanghai, China at a purity of 97.2%. For *in vitro* as well as *in vivo* analysis, the compound was dissolved in aqueous medium.

2.4 Determination of DPPH radical scavenging activity

DPPH scavenging activity was measured by the method of Aquino *et al* [9]. DPPH radical has an absorption peak at 515 nm, and the radical scavenging by an antioxidant will result in the change in absorption maximum by changing its colour from purple to yellow. DPPH in solution was incubated in the presence of curculigoside at concentrations ranging from 5-100 µg/ml for 20 minutes at room temperature in the dark and the absorbance was then read.

2.5 Determination of superoxide radical scavenging activity

NBT reduction method was performed according to the protocol of Mc Cord & Fridovich, [10] to determine the super oxide scavenging activity of curculigoside. Different concentrations of curculigoside (5-100 µg/ml) were added to the reaction mixture containing 1.5 mM NBT, 0.12 mM riboflavin, 0.067 M phosphate buffer (pH 7.8), EDTA/KCN and illuminated for 15 minutes, the absorbance was measured at 530 nm before and after illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes using the equation,

$$\{[\text{Control OD}-\text{Treated OD}]/\text{Control OD}\} \times 100$$

2.6 Determination of hydroxyl radical scavenging activity

Determination of hydroxyl radical (HO) scavenging activity was performed by Fenton reaction. Deoxy ribose is degraded by HO radicals which results in thiobarbituric acid reacting substance (TBARS) formation [11]. Different concentrations of curculigoside were mixed with the reaction mixture containing deoxyribose, FeCl₃, EDTA, H₂O₂, ascorbic acid, KH₂PO₄-KOH buffer and incubated for 1 h at 37°C. Percentage inhibition of HO radical formation was calculated by comparing absorbance of control tubes with that of treated tubes [12]. Vitamin C was used as positive control.

2.7 Determination of inhibition of lipid peroxidation

The lipid peroxidation was also determined by TBARS method [12]. Different concentrations of compound (1-100 µg/ml) was added to reaction mixture containing rat liver homogenate in Tris-HCl buffer, KCl, ferrous ion and ascorbic acid, incubated for 1 h at 37°C. The mixture (0.4 ml) was then treated with sodium dodecyl sulphate (SDS-8.1%), 0.8% thiobarbituric acid (TBA) and 20 % acetic acid. The percentage of lipid peroxidation inhibition was determined by comparing the results of the test compound with those of the control.

2.8 Determination of nitric oxide radical scavenging activity

NO released from sodium nitroprusside was estimated by Griess reagent method [13] To determine NO scavenging activity of curculigoside, sodium nitroprusside mixed with different concentrations of the compound (5-100 µg/ml) and incubated at 25°C for 15 min. Griess reagent was added and the absorbance was read at 546 nm and the percentage inhibition of nitric oxide formation was calculated by comparing with the control tube.

2.9 Determination of inhibition of nitric oxide produced by macrophages

Balb/c mice (male, 4-6 weeks old) were used for the studies in which macrophages were elicited by intraperitoneal injection of 5% sodium caseinate. Macrophages were collected from peritoneal cavity, washed and resuspended in complete RPMI-1640 medium containing 10% FBS to seed in a 96 well culture plate (1X10⁶ cells/well). After an incubation period of 2 h at 37°C, nonadherent cells were removed and adherent macrophages were treated with different concentrations of curculigoside (1-5 µg/ml) and then incubated for 24 h at 37°C with 5% CO₂. After 24 h the supernatant was collected for the estimation of nitric oxide by the Griess reaction [13]. The percentage inhibition of nitric oxide formation is calculated by comparing with the control.

2.10 Effect of Curculigoside on PMA induced superoxide radical generation in peritoneal macrophages

Balb/c mice (male, 4-6 weeks old) were divided in to two groups (4 animals/group) and injected with 5% sodium caesinate intra peritoneally to elicit macrophages. Group I animals were kept as control where as Group II animals were treated with five consecutive doses of curculigoside (20 mg/kg body weight) orally. After 1 h of the last dose of curculigoside administration, PMA (100 ng/animal) was injected to the animals to activate the peritoneal macrophages *in vivo*. Peritoneal macrophages were harvested after 3rd hour and the inhibition of superoxide generation in the macrophages by curculigoside was measured by NBT reduction method [14].

2.13 Statistical analysis

Values are expressed as mean \pm S.D. The statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. P-values less than 0.05 were considered to be significant.

3. Result

3.1 DPPH radical scavenging activity

Curculigoside was found to be an effective scavenger of DPPH radicals formed and the IC-50 (inhibitory concentration 50) value was 50 μ g/ml. Vitamin C, a known reducing agent showed an IC₅₀ value of 4.5 μ g/ml for the same reaction. The dose dependant inhibition on the DPPH radical formation by curculigoside is represented in Figure 1A.

3.2 Super oxide radical scavenging activity

Figure 1B represents the effect of curculigoside on super oxide radical formation by NBT reduction method. Curculigoside decreased the levels of superoxide formed by the reduction of NBT and the IC₅₀ value obtained was 86 μ g/ml where as the IC₅₀ value for the positive control vitamin C was 69 μ g/ml.

Figure 1: DPPH and Super oxide radical scavenging effect of curculigoside

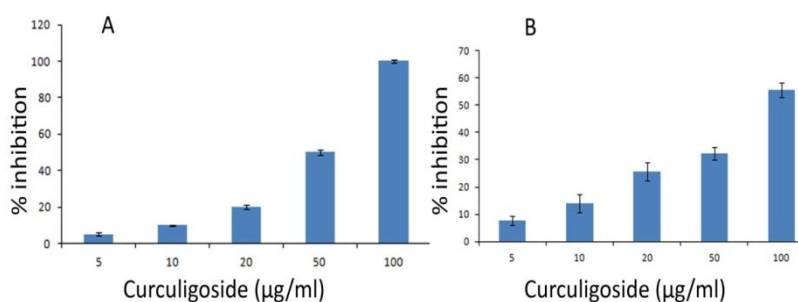


Figure 1 (A) Different concentrations of curculigoside were added to test system and determined the DPPH activity. 1(B). Superoxide scavenging effect of curculigoside was determined by NBT reduction method. The percentage inhibition was calculated with respective control tubes. Values are \pm SD of two different experiments.

3.3 Lipid peroxidation assay

Lipid peroxidation in the mouse liver homogenate was effectively scavenged by curculigoside (Figure 2A). IC₅₀ values obtained were 30 μ g/ml and 102 μ g/ml respectively for curculigoside and vitamin C when compared with the control tube.

3.4 Hydroxyl radical scavenging activity

A dose dependant scavenging effect on the hydroxyl radicals formation was exhibited by curculigoside with an IC₅₀ value of 37 μ g/ml (Figure 2B). Vitamin C was used as the positive control of HO scavenging assay and showed an IC₅₀ value of 86 μ g/ml.

Figure 2: Inhibitory effect of curculigoside on lipid peroxidation and hydroxyl radical formation

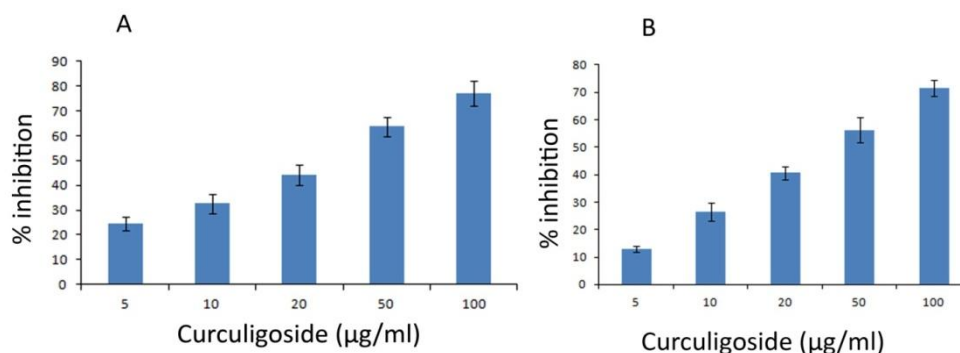


Figure 2(A) Effect of curculigoside on lipid peroxidation was determined by TBARS method. 2(B) Hydroxyl radical scavenging effect was determined by Fenton reaction. The percentage inhibition was calculated with respective control tubes. Values are \pm SD of two different experiments.

3.5 Nitric oxide scavenging activity

Curculigoside scavenged the NO released from sodium nitroprusside in the *in vitro* system. The dose dependant reduction of NO formation is represented in Figure 3 A. The compound was also found to be effective in reducing the NO release by cultured macrophages and the results are represented in Figure 3B. The NO release by the untreated control macrophages were 14.87 ± 1.62 which was significantly ($P < 0.01$) reduced to 7.4 ± 0.75 upon treatment with 5 μ g/ml curculigoside.

Figure 3: Nitric oxide scavenging effect of curculigoside

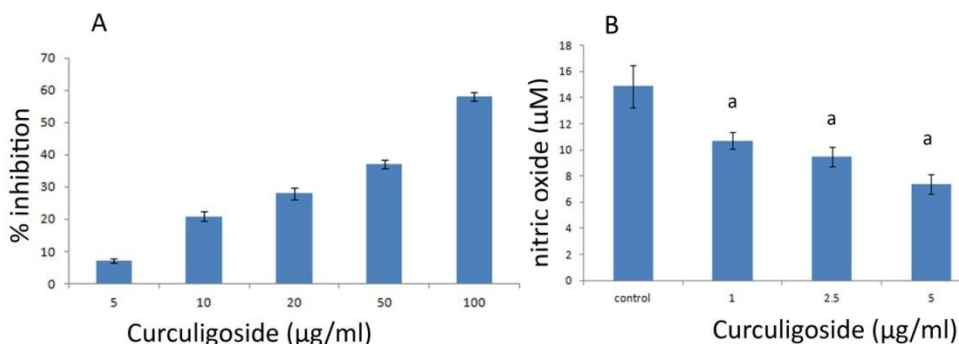


Figure 3(A) Effect of curculigoside on *in vitro* nitric oxide scavenging activity. 3(B) Effect of curculigoside on NO release by mice peritoneal macrophages. % inhibition was calculated by comparing with the control. Values are expressed as \pm SD. $aP < 0.01$, when compared to control.

3.6 Effect of curculigoside on PMA induced superoxide radical formation

In vivo superoxide scavenging activity of curculigoside was determined by PMA induced superoxide production method. Oral administration of curculigoside at a concentration of 20 mg/ml reduced the superoxide radical generated significantly ($P < 0.01$) during the activation with PMA in sodium caseinate induced macrophages with a percentage inhibition of 50.6 % (Table 1).

Table 1: Effect of curculigoside on PMA induced superoxide generation in peritoneal macrophages

Treatment	OD	% inhibition
Control	0.67 ± 0.02	---
Curculigoside	$0.33 \pm 0.03^{**}$	50.6 %

Data are represented as mean \pm SD. Data were analyzed by One-way ANOVA followed by Dunnett's test. $**P < 0.01$ when compared to the control animals.

4. Discussion

Free radical and reactive oxygen species are the by-products of oxidative metabolism taking place in the cells. Reactive oxygen species may play a role in the pathogenesis of cancer [15,16] and in other diseases including inflammation, bacterial infections, AIDS, etc [17]. According to Halliwell & Gutteridge antioxidants are 'any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate' [18]. Due to the unhealthy side effects of synthetic antioxidants, natural antioxidants are getting more attention now a day [19]. Traditionally used well known natural antioxidants include green tea, antioxidants from fruits, vegetables, spices etc and some antioxidants like rosemary, sage etc are already exploited commercially either as antioxidant additives or as nutritional supplements [20,21].

Lipid peroxidation is the oxidative degradation of lipids by reactive oxygen species such as hydroxyl radical, hydrogen peroxide etc. naturally occurs in the body. Polyunsaturated fatty acids of the biological membranes are the major targets of these reactive oxygen species attack. The chain reaction of lipid peroxidation and its end-products can affect the viability of cells, even tissues [22]. Curculigoside was found to inhibit lipid peroxidation significantly during the *in vitro* studies. In the case of hydroxyl radical scavenging effects curculigoside resulted in a far better results than vitamin C, a known antioxidant. This strongly suggests the potential of curculigoside as free radical scavenging agent.

Super oxide free radicals are extremely toxic to living system and generated in large quantities during the enzymatic oxidation of NADPH. Curculigoside was found exhibit good scavenging effect on the super oxide radicals generated by the reduction of NBT. Macrophages generate superoxide radicals as an immune defence mechanism. Oral administration of curculigoside (20 mg/kg b.wt) in Balb/c mice reduced the super oxide radical production by the peritoneal macrophages elicited by PMA.

DPPH assay is one of the most convenient and stable assay to estimate *in vitro* antioxidant activity. From our studies curculigoside was found to exhibit DPPH scavenging activity comparable with that of ascorbic acid. A concentration of 100 µg/ml resulted in 60% inhibition where as 82 % inhibition was obtained for vitamin C on DPPH radicals production.

Nitric oxides are free radicals with a number of biological activities. At low concentration it acts as a biological signalling molecule and vasodilator [23]. At higher concentrations they are capable to produce enormous biological damages. Nitric oxide scavenging activity of curculigoside was estimated by *in vitro* tube test and also in the cultured macrophages. Macrophages releases NO as a part of immune response which is toxic to micro organisms at the same time,

enhanced levels of NO initiates inflammatory responses and can initiate biological damages [24].

Thus it is concluded that, Curculigoside, a natural phenolic derivative from the plant *Curculigo orchioides* is found to exhibit profound antioxidant effect both in the *in vitro* and *in vivo* systems. In the case of hydroxyl radical scavenging and inhibition of lipid peroxidation, curculigoside gave better results than vitamin C, where as in the case of DPPH and super oxide radicals the scavenging effect was more prominent for vitamin C.

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Conflict of interest: The authors report no conflict of interest.

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