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Research Article

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In vitro antioxidant activity of cholestanol glucoside from an endophytic fungus *Lasiodiplodia theobromae* isolated from *Saraca asoca*

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ABSTRACT

Nature remains to be an attractive source of bioactive compounds and natural compounds are extensively being explored for their therapeutic applications. Steroidal glycosides are a major group of secondary metabolites that exhibit broad spectrum of biological activities. Several steroidal glycosides have been reported to possess promising anti-oxidant activities. In the present study we investigate the anti-oxidant potentialities of cholestanol glucoside (CG), a steroidal glycoside produced by Lasiodiplodia theobromae, an endophytic fungus isolated from Saraca asoca, using in vitro assays such as DPPH radical scavenging assay, nitric oxide radical scavenging assay, hydroxyl radical scavenging assay, super oxide radical scavenging assay and hydrogen peroxide scavenging assay. The compound showed significant free radical scavenging capacity with an IC_{50} value of 16.2 µM. The compound could also effectively scavenge hydrogen peroxide (IC_{50} value of 7.2 µM) and hydroxyl radicals (IC_{50} value of 3.6 µM).

Keywords: Anti-oxidant, cholestanol glucoside, endophytic fungus, *Lasiodiplodia theobromae*, *Saraca asoca.*

INTRODUCTION

Free radicals are generated in living systems during respiration, phagocytosis, prostaglandin synthesis and also by cytochrome P450 system [1]. In addition to the free radicals generated in the body as a part of cellular metabolism, they can also result from external factors such as pollution, cigarette smoke, radiation and medication. This overload of free radicals becomes deleterious to cells [2]. Human body possesses enzymatic and non enzymatic endogenous antioxidant systems that can neutralize the reactive oxygen and reactive nitrogen species [3]. The major antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. The non enzymatic endogenous antioxidants are lipoic acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins and transferrin. Several exogenous antioxidants present in our diet can work in coordination with the endogenous antioxidant system to detoxify the free radicals and maintain the body healthy [4].

Recent researches suggest that dietary antioxidants play an essential role in the prevention of cardiovascular diseases [5], cancers [6], neurodegenerative diseases including Parkinson's and Alzheimer's diseases [7] as well as inflammation and cutaneous aging [8]. Natural compounds like phenolic acids, polyphenols, flavanoids, carotenoids, ascorbic acid, tocopherol, omega-3-fatty acids and omega-6-fatty acids are reported to have high free radical scavenging activity [9,10]. Saponins are a class of natural glycosides with diverse biological activities [11]. Several steroidal glycosides such as glycosidic derivatives of hecogenin, diosgenin, gitogenin and digitogenin have been reported as potent antioxidants [12]. In the present study we report the in vitro antioxidant activity of cholestanol glucoside (CG), a steroidal saponin produced by *Lasiodiplodia theobromae*, an endophytic fungus isolated from

Saraca asoca. Cholestanol glycosides have been reported as antitumor agents [13, 14, 15]. However, the antioxidant and cytoprotective effects of cholestanol sugars remain largely underexplored. Our study suggests cholestanol glucoside as a promising lead structure for the development of cytoprotective agents.

EXPERIMENTAL SECTION

Chemicals used in the study

1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), sodium nitroprusside, Griess reagent, ascorbic acid, riboflavin, hydrogen peroxide and 2-deoxy ribose were purchased from Sigma Aldrich. Ferrous sulphate, ethylene diamine tetraacetic acid (EDTA), sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merck, Mumbai, India. Nitro blue tetrazolium (NBT) and trichloroacetic acid (TCA) were purchased from SD fine chemicals, India.

DPPH radical scavenging assay

The antioxidant activity of CG was determined by DPPH radical scavenging assay [16]. 1.8, 3.6, 9, 13.5,18, 22.5 and 27 µM concentrations of CG (dissolved in 0.5 ml of methanol) was added to 1 ml of 0.1 mM methanolic solution of DPPH. The reaction mixture was incubated at room temperature in dark for 30 minutes. The DPPH radical scavenging activity was determined at 517 nm. Methanol was used as the blank. The control contained all reagents except CG. The experiments were carried out in triplicate. Ascorbic acid was used as the positive control.
The DPPH radical scavenging activity was calculated using the following formula,

$$\% \, Inhibition = \frac{A0 - A1}{A0} * 100$$

where A0 was the absorbance of the control and A1 was the absorbance of the sample.

Nitric oxide radical scavenging assay

Nitric oxide production from sodium nitroprusside was measured according to Dayana et al [17]. The assay mixture contained 0.3 ml of sodium nitroprusside (10 mM) in phosphate-buffered saline (pH 7.4), mixed with 1 ml of CG (1.8, 3.6, 9, 13.5, 18, 22.5 and 27 µM) in sodium phosphate buffer. The volume of reaction mixture was made upto 3 ml using sodium phosphate buffer and incubated at room temperature for 150 min. The same reaction mixture, without CG but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent was added to 2.5 ml of reaction mixture. The absorbance of the chromophore formed was read at 546 nm.

Nitric oxide scavenging activity was calculated using the formula,

$$\% \, Inhibition = \frac{A0 - A1}{A0} * 100$$

where A0 was the absorbance of the control and A1 was the absorbance of the sample.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging ability of CG was determined according to the method of Smirnoff et al [18]. Briefly, the reaction mixture consisted of 0.2 ml each of 10 mM $FeSO_4.7H_2O$, 10 mM EDTA, 10 mM 2-deoxy ribose, different concentrations (1.2 ml) of CG (1.8, 3.6, 9, 13.5, 18, 22.5 and 27 µM) and 0.2 ml 10mM H_2O_2. Reaction mixture without CG served as the control. After incubation for 4 hours at 37°C, 1ml each of 2.8 % TCA and 1% TBA were added and the mixture was incubated at 90°C for 10 min to develop color. After cooling, the mixture was centrifuged (400 g, 5 min) and the absorbance was read at 532 nm.

Scavenging activity was calculated using the formula,

$$\% \, Inhibition = \frac{A0 - A1}{A0} * 100$$

where A0 was the absorbance of the control and A1 was the absorbance of the sample.

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging assay was done using the method as described by Rush et al. [19]. The assay is based on the decrease in absorbance of H_2O_2 upon oxidation. CG at different concentrations (1.8, 3.6, 9, 13.5, 18, 22.5 and 27 µM respectively) dissolved in 3.4 ml of 0.1 M sodium phosphate buffer was allowed to react with 0.6 ml of 40 mM H_2O_2 for 30 min. Absorbance of the reaction mixture was read at 230 nm. 0.1 M sodium phosphate buffer was used as blank and the reaction mixture without CG was used as the control.

Hydrogen peroxide scavenging activity was calculated using the formula,

$$\% \, Inhibition = \frac{A0 - A1}{A0} * 100$$

where A0 was the absorbance of the control and A1 was the absorbance of the sample.

Superoxide anion radical scavenging assay

Superoxide anion scavenging activity of cholestanol glucoside was measured by the method of Beauchamp et al [20]. The reaction mixture in 2 ml 50 mM sodium phosphate buffer (pH 7.6) contained 20 µg riboflavin, 12 mM EDTA, 0.6 ml of NBT solution (1 mg/ml) and CG at concentrations 1.8, 3.6, 9, 13.5, 18, 22.5 and 27 µM respectively. The reaction solution was illuminated under fluorescent lamp for 15 min and the absorbance at 560 nm was measured using a spectrophotometer. Reaction mixture kept in dark and served as blank. The percentage inhibition of superoxide anion generation was calculated as

$$\% \, Inhibition = \frac{A0 - A1}{A0} * 100$$

where A0 was the absorbance of the control, and A1 was the absorbance of the sample.

Statistical analysis

Experimental results were mean ± SD of three parallel measurements. The data were analysed by an analysis of variance ($p < 0.05$).

RESULTS AND DISCUSSION

DPPH free radical scavenging activity

The antioxidant activity of CG was tested with DPPH scavenging assay which is based on the reduction in the absorption maximum of DPPH radicals in presence of an antioxidant compound. Ascorbic acid was chosen as the standard antioxidant. The results of DPPH radical scavenging activity of CG is shown in Figure 1. The compound exhibited a concentration dependent increase in free radical scavenging activity. Although the compound exhibited 50% scavenging activity only at a concentration of 16.2 µM, the scavenging activity approaches 100% at 27 µM.

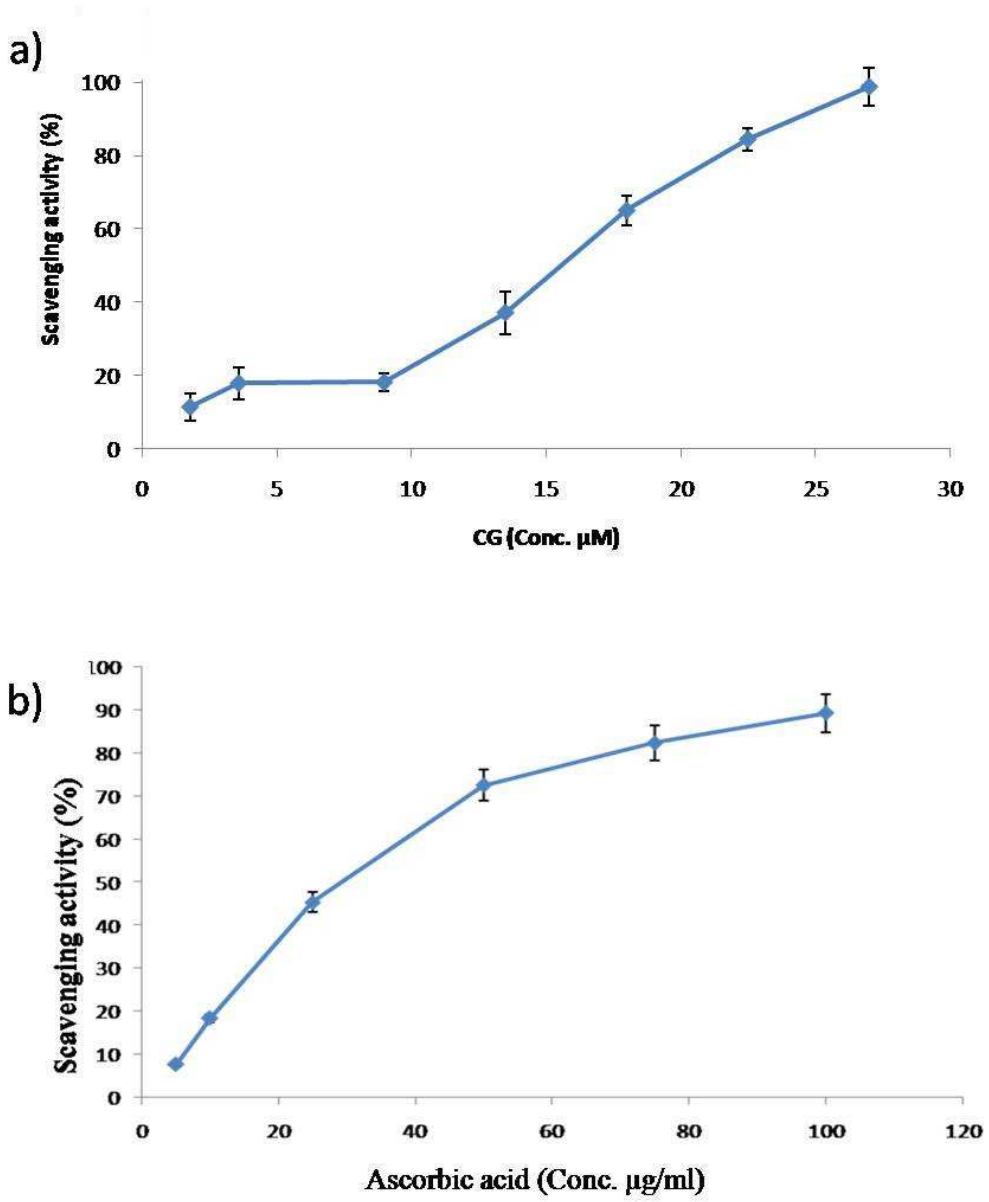

Figure 1. DPPH radical scavenging activity of CG (a) and the positive control ascorbic acid (b).
All values are reported as mean ±SD (N=3).

Nitric oxide scavenging activity

CG did not have significant nitric oxide scavenging activity when compared to the standard, ascorbic acid. Although CG exhibited 48% scavenging activity at 18 µM, no significant increase in activity could be obtained at higher concentrations (Figure 2). The positive control used in the assay, ascorbic acid exhibited nitric oxide scavenging activity of 68% at a concentration of 100 µg mL^{-1}.

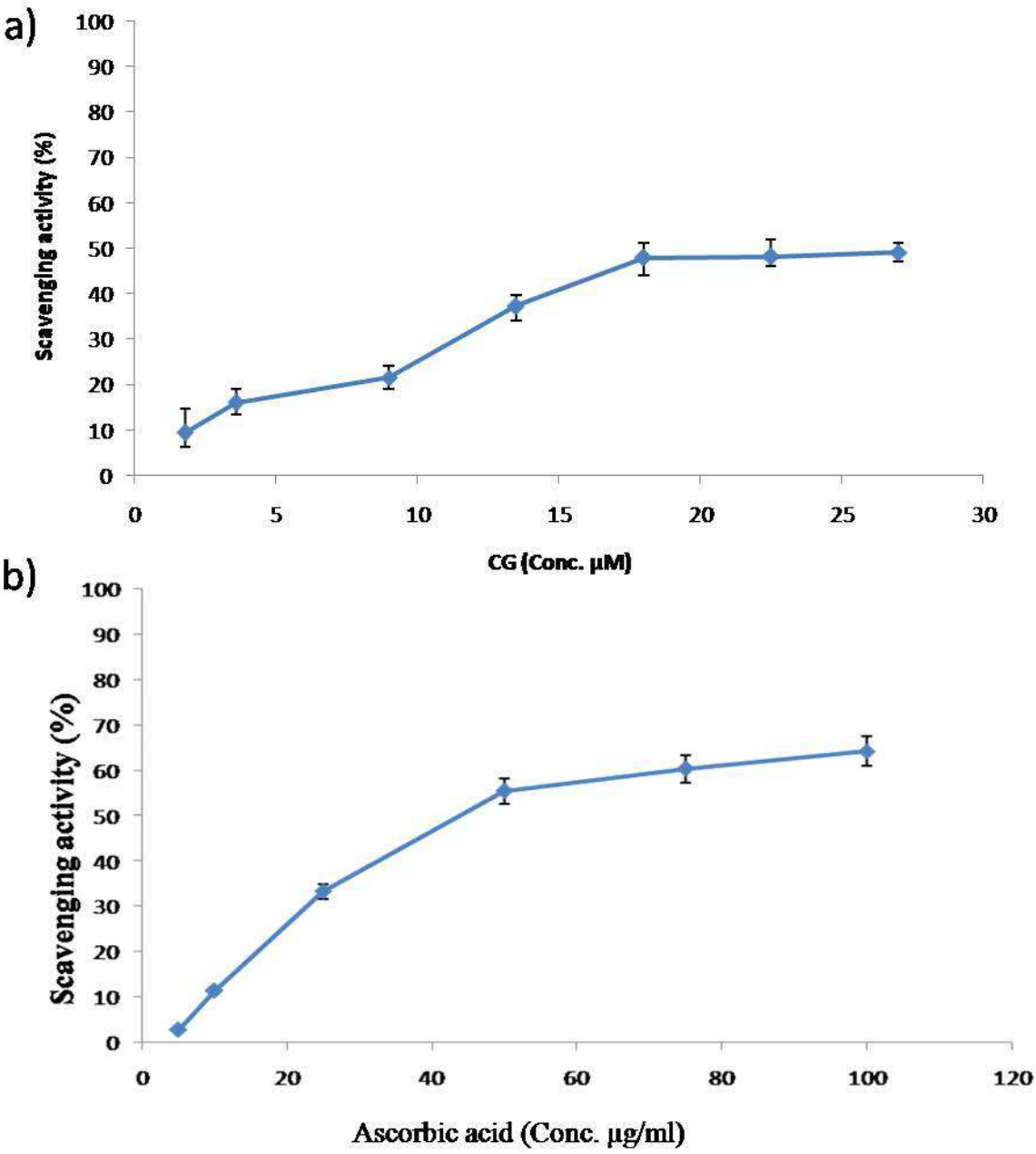

Figure 2. Nitric oxide radical scavenging activity of CG (a) and the positive control ascorbic acid (b).
All values are reported as mean ±SD (N=3).

Hydroxyl radical scavenging activity

CG exhibited remarkable hydroxyl radical scavenging activity with an IC_{50} value of 3.6 µM (Figure 3). Although a dose dependent increase was exhibited by the compound, the activity obtained at the highest concentration (27µM) was 72%.

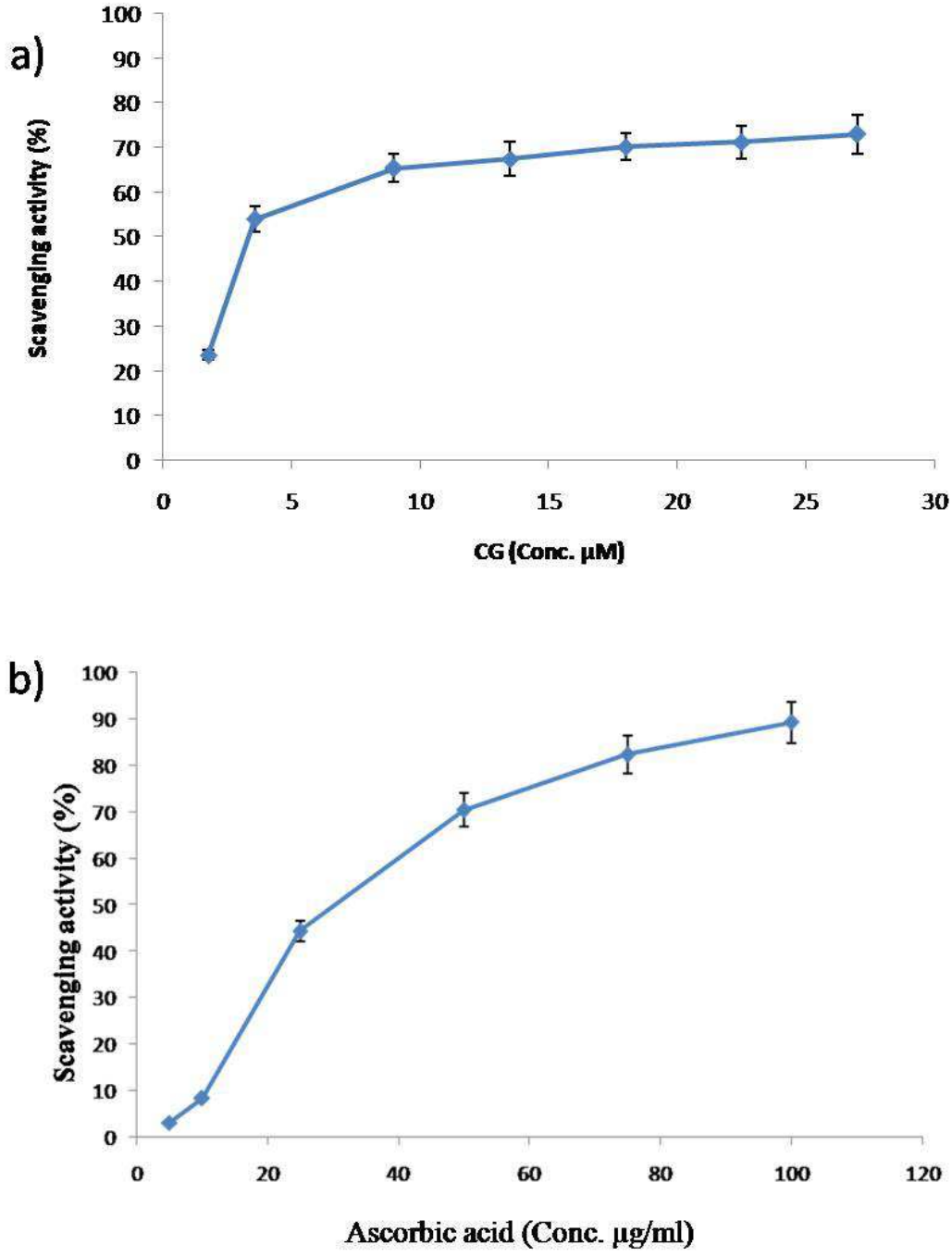

Figure 3. Hydroxyl radical scavenging activity of CG (a) and the positive control ascorbic acid (b).
All values are reported as mean ±SD (N=3).

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of CG is given in Figure 4. The compound exhibited significant H_2O_2 scavenging activity with an IC_{50} value of 7.2 µM as compared to that of ascorbic acid having an IC_{50} value of 32 µg ml^{-1}. The activity increased with increasing concentrations of CG with the scavenging ability approaching 98% at 27 µM.

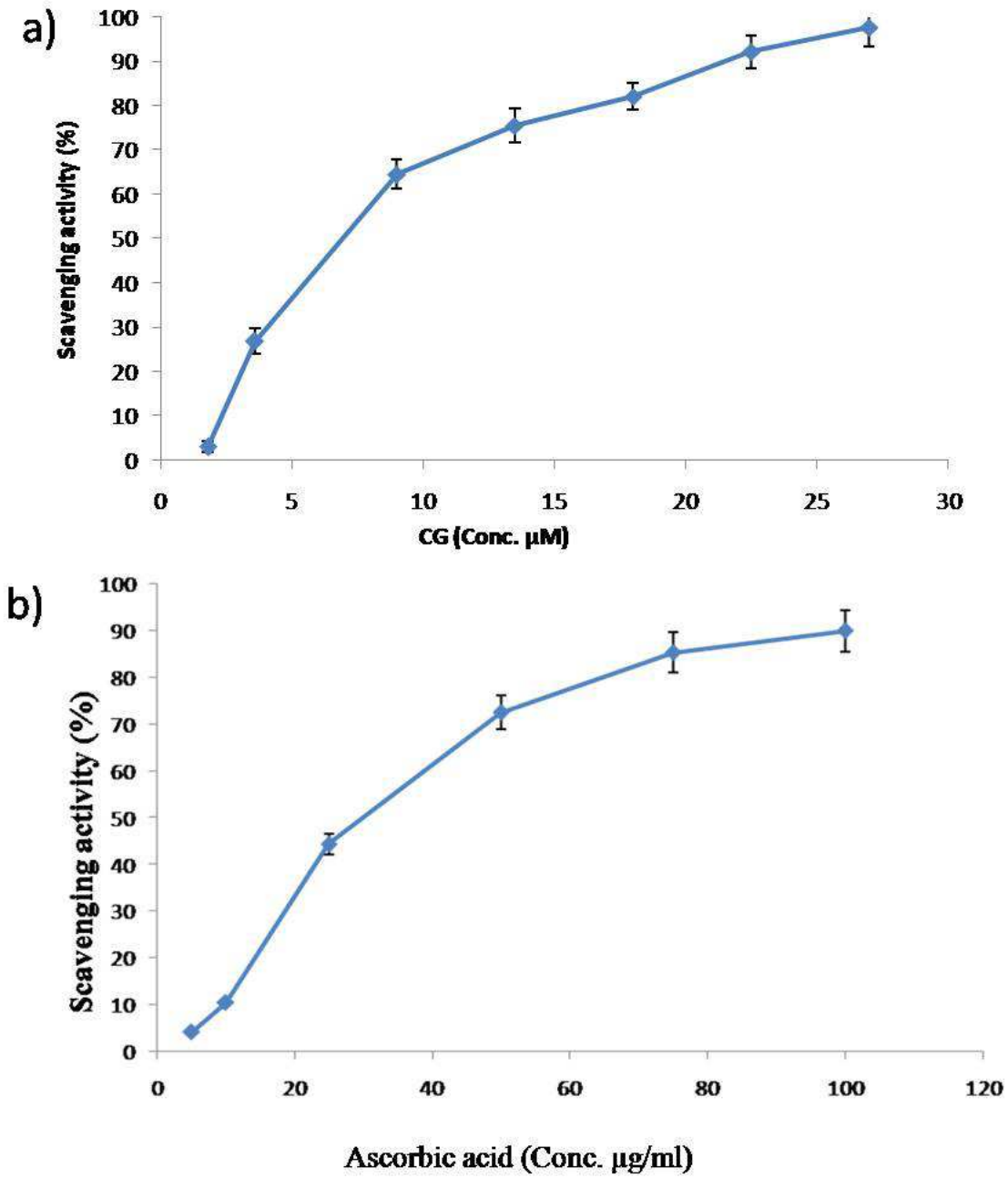

Figure 4. Hydrogen peroxide scavenging activity of CG (a) and the positive control ascorbic acid (b).
All values are reported as mean ±SD (N=3).

Superoxide anion radical scavenging activity

The superoxide anion scavenging activity of CG was studied at different concentrations ranging from 1.8 to 27 µM. The assay involves a non-enzymatic generation of superoxide anions. The superoxide anion scavenging activity was determined by measuring the reduction in the rate of formation of formozan dye, which is blue in color and absorbs at 560 nm. CG had poor superoxide scavenging activity as shown in Figure 5. The compound was largely inactive against superoxide radicals. The compound had only 24% scavenging capacity even at 18 µM. Ascorbic acid was found to scavenge superoxide anions with an IC_{50} value of 25 µg ml^{-1}.

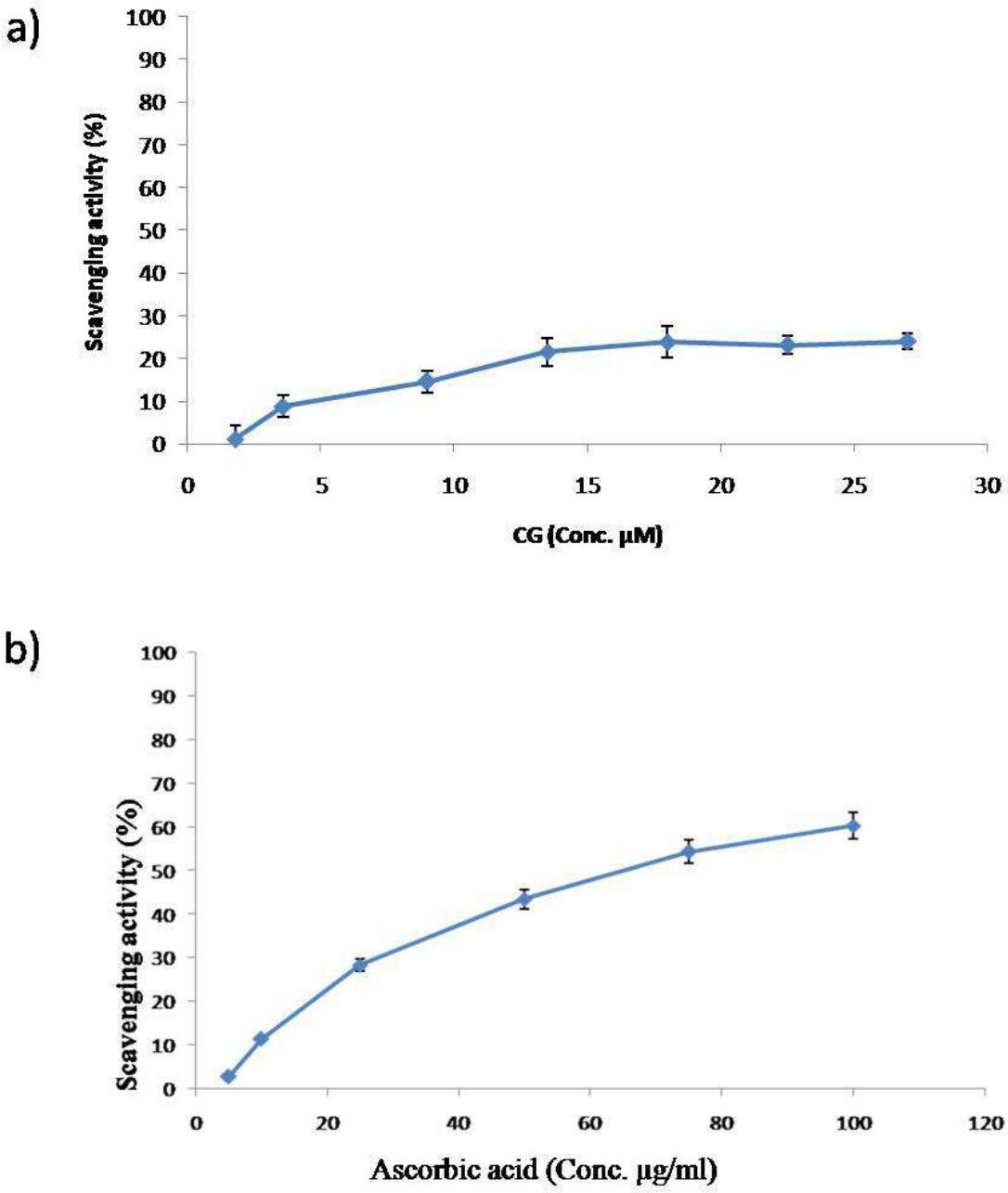

Figure 5. Superoxide anion scavenging activity of CG (a) and the positive control ascorbic acid (b).
All values are reported as mean ±SD (N=3).

CONCLUSION

Oxidative stress in cells is reported to have serious implications in many chronic diseases such as cancer, heart diseases and neurodegenerative diseases. In addition to the free radicals generated in cells as a part of cellular metabolism, excessive free radicals resulting from external factors can produce oxidative stress in cells. In such cases it is essential to augment the endogenous antioxidant system by supplementation of exogenous antioxidants. Many secondary metabolites are known to be efficient scavengers of free radicals. Several steroidal glycosides are known to have antioxidant properties. In the present study, we have attempted to study the in vitro antioxidant potentialities of a steroidal glycoside, cholestanol glucoside. Standard assays such as DPPH free radical scavenging

assay, nitric oxide radical scavenging assay, hydrogen peroxide scavenging assay, hydroxyl radical scavenging assay and superoxide anion scavenging assay were used. The compound had significant free radical scavenging capacity as evident from the concentration dependent reduction in absorbance of DPPH radicals at 517 nm. The compound had poor nitric oxide and superoxide scavenging activities. The compound could efficiently scavenge hydrogen peroxide (IC_{50} value of 7.2 µM) and hydroxyl radicals (IC_{50} value of 3.6 µM). The antioxidant activity of *L. theobromae* derived CG was comparable to that of ascorbic acid used as the positive control. The results suggest CG to be a promising therapeutic agent in preventing or slowing the progress of aging and age related oxidative stress induced degenerative diseases. Moreover, the fungal origin of the compound ensures a sustained supply and economic feasibility of production. However, the antioxidant activity and efficacy of the compound has to be evaluated in vivo.

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