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In vitro antioxidant activity of *Holarrhena antidysenterica* Wall. methanolic leaf extract

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ABSTRACT
Antioxidative potential of methanolic leaf extract of *Holarrhena antidysenterica* was evaluated using hydroxyl radical, superoxide anion scavenging and reducing power assays. The antioxidant activity of the methanol extract increased in a concentration-dependent manner. The extract showed significant reactive oxygen species (ROS) scavenging activity in all *in vitro* antioxidant assays and contained high level of total phenolic content.

KEY WORDS: Medicinal plant, antioxidant, reactive oxygen species, nutraceutical

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INTRODUCTION

Reactive oxygen species (ROS), which consist of free radicals such as superoxide anion (O2·–) and hydroxyl (HO·) radicals and non-free radical species such as H_2O_2 and singled oxygen ($^1O_2$), are different forms of activated oxygen [1, 2, 3]. ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA. Thus, ample generation of ROS proceed to a variety of pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity [4, 5]. Therefore, living organisms possess a number of protective mechanisms against the oxidative stress and toxic effects of ROS.

Antioxidants regulate various oxidative reactions naturally occurring in tissues and are evaluated as a potential anti-aging agent. Hence, antioxidants can terminate or retard the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as electron donors. Antioxidants have been widely used as food additives to provide protection from oxidative degradation of foods and oils. Hence, antioxidants are used to protect food quality mainly by the prevention of oxidative deterioration of constituents of lipids. The most extensively used synthetic antioxidants are propylgallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) [6]. However BHT and BHA have been suspected of being responsible for liver damage and carcinogenesis [7-9]. Natural antioxidants are able to protect from ROS as well as other free radicals and retard the progress of many chronic diseases and lipid oxidative rancidity in foods [10- 13].

Holarrhena antidysenterica Wall. (Apocynaceae), commonly known as "Kutaja", is an important plant used in indigenous systems of medicine as remedy for bronchitis, hematuria, spermatorrhoea, epilepsy, asthma, piles, leprosy, eczema, diarrhea, fevers and jaundice [14, 15]. The bark has been reported to possess astringent and antidiarrheal properties [16]. Leaves of the plant are used to cure scabies [17]. In addition, the leaves of *H. antidysenterica* have been reported to have antibacterial, anti-inflammatory and analgesic activity [18, 19].

Nevertheless, there is as yet no report concerning the antioxidant effect of this plant. Our main objective is to determine the potential natural antioxidant source. However, the purpose of this particular study was to evaluate the *in vitro* antioxidant activities of methanol extract of *H. antidysenterica*.

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MATERIALS AND METHODS

Plant materials:

Fresh material of plant in the flowering stage above 20 cm diameter at breast height were collected locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in May 2006. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR204).

Extraction:

Freshly collected leaves of *H. antidysenterica* were shade-dried and then powdered using a mechanical grinder. 250 grams of pulverized leaf material were soaked in 750 ml of methanol (LR grade, Merck, India) and kept on a rotary shaker for 24 h. Each extract was filtered under vacuum through a Whatman No. 1 filter paper and the process repeated until all soluble compounds had been extracted. Extraction was considered to be complete when the filtrate had a faint colour. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). A portion of the residue was subjected to screening for antioxidant activity.

ANTIOXIDANT ACTIVITY

Superoxide anion scavenging activity:

Superoxide anion scavenging capacity of *H. antidysenterica* was assessed by the method of Nishikimi [20]. About 1 mL of reaction mixture contained 156 µM nitoblue tetrazolium in 100 mM phosphate buffer (pH 7.4), 468µM NADH in 100 mM phosphate buffer (pH 7.4) with 0.1 mL of extract (200µg to 1000µg as equivalents to tannic acid) in methanol were mixed. The reaction started by adding 100 µL of 60 µM phenazine methosulphate solution in 100mM phosphate buffer (pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min, correspondingly blank contained all the reagents except extract of *H. antidysenterica* and the absorbance of oxidized product formazan was recorded at 560 nm. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage of super oxide anion scavenging was calculated as:

$$\% \text{ scavenged of superoxide anion} = [(A_{blank} - A_{Sample}) / A_{blank}] \times 100$$

where A_{blank} is the absorbance of the blank in absence of sample, and A_{Sample} is the absorbance in the presence of the sample.

Hydroxyl radical scavenging assay:

The ability of the extract of *H. antidysenterica* to scavenge the hydroxyl radical being generated by Fenton reaction Fe^{3+}–ascorbate–EDTA–H_2O_2 system was performed by the method of Halliwell *et al.* [21] with minor changes. Freshly prepared 1.0ml of final reaction mixture containing 100µl of 28mM 2-deoxyribose in phosphate buffer (pH 7.4), 500µl of various concentrations of (100–500µg/ml) *H. antidysenterica* extract, 200µl of 200µM $FeCl_3$ in 1.04mM EDTA (1:1, v/v), 100µl each of 1.0mM H_2O_2 and ascorbic acid, respectively. After 1hr of incubation at 37°C the free radical damage imposed on deoxyribose was measured as TBARS by the method of Ohkawa *et al.* [22] 1.0 ml thiobarbituric acid (1%) and 1.0ml of trichloro acetic acid (2.8%) were added to the test tubes and were incubated in boiling water bath for 20min. After cooling, absorbance was measured at 532nm against the blank containing deoxyribose and buffer. The percentage of hydroxyl radical scavenging was calculated as:

$$\% \text{ Scavenged } [H_2O_2] = [(A_{blank} - A_{Sample}) / A_{blank}] \times 100$$

where, A_{blank} is the absorbance of the blank in absence of sample, and A_{Sample} is the absorbance in the presence of sample.

Fe^{3+}- Reducing power assay:

The Fe^{3+}-reducing power was determined using the method described by Oyaizu [23]. Different concentrations (50–250µg as equivalents to tannic acid) of *H. antidysenterica* extract in 1ml of methanol were mixed with 2.5 ml of phosphate buffer (200mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was placed in a water bath for 20 min at 50°C, then cooled rapidly and mixed with 2.5 ml of 10% trichloroacetic acid. The resulting solution was centrifuged at 3000 rpm for 10 min. From the supernatant 2.5ml of fraction was mixed with 2.5ml of distilled water and 0.5 ml of 0.1% ferric chloride and the amount of iron(II)-ferricyanide complex could be determined by measuring the formation of Perl's Prussian blue at 700 nm after 10 min. The higher absorbance of the reaction mixture indicates increased reducing power.

Total phenolic assay:

Total phenolic in the methanolic leaf extract of *H. antidysenterica* was determined according to the method of Shahidi and Naczk [24]. An amount of 0.25 ml aliquot of the prepared samples were mixed with 0.25 ml Folin-Ciocalteu reagent (previously diluted with water 1:1 (v/v)) and 0.5ml of saturated sodium carbonate (Na_2CO_3) solution and 4ml of deionized water. The mixtures were intensively shaken, left at room temperature for 25 min and centrifuged at 5000 rpm for 10 min. the supernatant absorbance was measured at 725 nm using spectrophotometer. The results were expressed as tannic acid equivalents in milligram per gram of the extract, using a standard curve generated with tannic acid and calculation was carried out by using an equation. The equation is given below:

$$\text{Absorbance} = 0.001 \times \text{tannic acid } (\mu g) + 0.0033$$

RESULTS

The superoxide anion derived from dissolved oxygen by phenazine methosulphate/NADH coupling reaction reduces nitro blue tetrazolium. The decrease in the absorbance at 560 nm with the plant extract thus indicates the consumption of superoxide anion in the reaction mixture. As depicted in Fig. 1, the methanolic extract possesses the scavenging activity with efficacy of 50% inhibition at 500µg/ml as equivalents to tannic acid.

To attack the substrate deoxyribose, hydroxyl radicals were generated by the reaction of Fe^{3+}–EDTA together with H_2O_2 and ascorbic acid. When the plant extract was incubated with the above reaction mixture, it could prevent the damage against the sugar. The results are shown in Fig. 2, the required concentration for 50% inhibition was found to be 285µg/ml as equivalents to tannic acid.

Fig. 3 shows the reductive capabilities of the plant extract. The reducing power of the methanolic extract of *H. antidysenterica* as shown in figure there is a dose-dependent increase in absorbance. This indicates that, alcoholic fraction of *H. antidysenterica* plant extract could reduce the most Fe^{3+} to Fe^{2+}ions.

The total phenolic content of alcoholic extract of *H. antidysenterica* was expressed as 41.32 mg of tannic acid equivalents per gram of extract.

DISCUSSION

Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. It has been proved that these mechanisms may be important in the pathogenesis of certain diseases and ageing. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the progress of complications associated with diseases [25]. Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidant activity [26]. Flavonoids and other phenolic compounds (hydroxyl cinnamic derivatives, catechines, etc.) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation [27].

In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduced NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. In the present study, the methanolic extract of *H. antidysenterica* has shown a marked activity and absorbance values shown that there is dose dependent superoxide anion scavenging activity. The extract was examined for its ability to act as OH• scavenging agent. Fe^{3+}–EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4; free hydroxyl radicals formed in solution and were detected by their ability to degrade 2-deoxyribose into fragments, upon heating with TBA at low pH form a pink chromogen [20]. While *H. antidysenterica* extract was added to the reaction mixture the hydroxyl radicals were removed and prevented the degradation of 2-deoxyribose as mentioned above. The phenolic compounds present in the extract may contribute directly to antioxida-

Figure 1: Effect of H. antidysenterica methanolic extract on scavenging of super oxide anion radical.

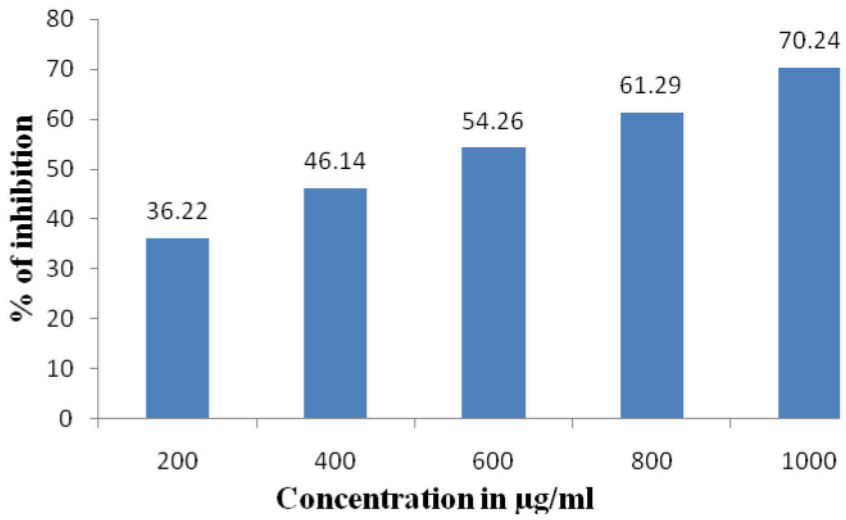


Figure 2: Scavenging effect of methanolic extract from H. antidysenterica on hydroxyl radical.

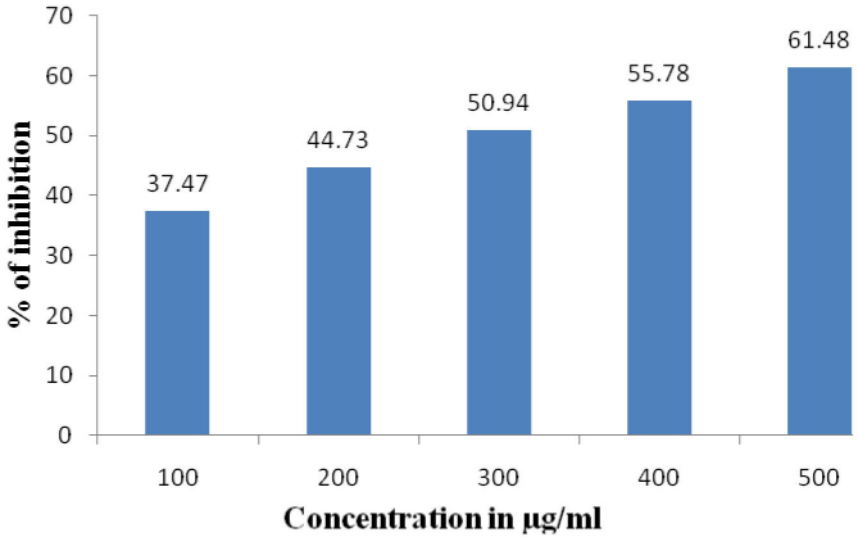
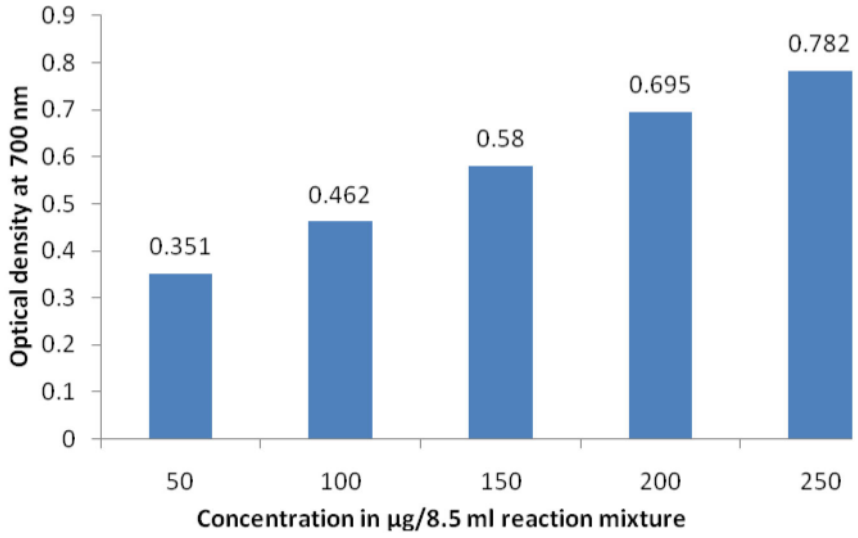


Figure 3: Reductive ability of the methanolic extract from *H. antidysenterica.*



tive action [28]. These results indicate that polyphenols present in extract could be responsible for the beneficial effects. For the measurement of reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of methanolic extract. The reducing power of the methanolic extract of *H. antidysenterica* increases with the increased concentration of the methanolic extract of *H. antidysenterica*. Compelling evidences indicate that methanolic extract of *H. antidysenterica* stem bark contain high concentration of phenolic content. Previously reported literature indicated the significance of phenolic compounds in preventing the ROS effect [29-32]. Interestingly in our study we have found higher concentration of phenolic compounds in the tested methanolic extract of *H. antidysenterica*. This phenolic concentration may be responsible for the antioxidant potency of the extract against different free radicals generated.

CONCLUSION

This study suggested that the *H. antidysenterica* plant extract possess antioxidant activity, which might be helpful in preventing or slowing the progress of various oxidative stress- related diseases. Further investigation on the isolation and identification of antioxidant component(s) in the plant may lead to chemical entities with potential for clinical use.

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