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Evaluation of antioxidant activities and total phenolic content of methanolic extract of *Rhodiola heterodanta* roots

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ABSTRACT

Rhodiola heterodanta belong to the stone crop family Crassulaceae, is an important medicinal plant and food crop in Trans-Himalayan cold desert. It is a well-known medicinal plant in the Amchi system of medicine (Tibetan system of medicine) being used in various ailments like anti-stress, anticancer, radio-protective, adaptogens, anti-inflammatory agent etc. Methanolic extract of *R. heterodanta* root was investigated in the present study to quantify total Phenolic content as well as anti-oxidant activity. Determination of total phenolic content of the methanol extract of *R. heterodanta* was achieved using Folin–Ciocalteau reagent. It was found to be 179.42±.63 mg GAE/g plant extract. The DPPH activity of the extract (0.05–0.5 mg/ml) was increased in a dose dependent manner, which was found in the range of (25.38-73.69%) as compared to ascorbic acid (31.48 – 77.17%). The IC_{50} values of methanol extract in DPPH radical, hydroxyl radical, nitric oxide radical and superoxide radical were obtained to be 0.28, 0.27, 0.28, 0.26 mg/ml, respectively. However, the IC_{50} values for the standard ascorbic acid were noted to be 0.21, 0.24, 0.22., 0.19 mg/ml, respectively. These results clearly indicate that *R. heterodanta* is effective against free radical mediated diseases and also helpful to draw special attention for further studies.

Keywords: *Rhodiola heterodanta;* Total polyphenolic; Anti-oxidant; Methanolic root extract.

1. Introduction

In recent years much attention has been devoted to natural anti-oxidant and their association with health benefits. Plants are potential source of natural antioxidants [1]. It produces various antioxidant compounds to counteract reactive oxygen species (ROS) in order to survive. ROS viz. superoxide radicals, hydroxyl radicals, and hydrogen peroxide are generated as by-products of biological reactions during normal cell aerobic respiration. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals and other ROS are also cause great damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity and DNA mutations leading to cancer, atherosclerosis, hypertension and other diseases [2]. The phenolic compounds in herbs act as antioxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators [3]. Anti-oxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol derived radical to stabilise and delocalise the unpaired electron (chain breaking function), and from their ability to chelate transition metal ions [4]. The protective action of medicinal plants has been attributed to the presence of antioxidants, especially polyphenolic compounds and antioxidant vitamins, including ascorbic acid, tocopherol, b-carotene, flavonoids, tannins, anthocyanins, and other phenolic constituents [5, 6]. Food rich in antioxidants plays an essential role in the prevention of cardiovascular diseases and cancers [7, 8] and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases [9]. Thus, it is important to increase the antioxidant intake in the diet and search for natural antioxidant sources among plants used as food additives. Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) commonly used in processed foods have side effects and are carcinogenic. In recent years, the use of natural antioxidants present in food and other biological materials has attracted considerable interest due to their presumed safety, nutritional and therapeutic value [10].

Rhodiola heterodanta (Crassulaceae) commonly known as golden root, is a perennial plant with a thick rhizome. It is also an important food crop and medicinal plant in Trans-Himalayan cold desert [11]. Root extracts are the excellent source of important commercial preparations widely used throughout Europe, Asia and more recently in the USA, with biological activities including antiallergenic and anti-inflammatory effects, enhanced mental alertness, and a variety of therapeutic applications [12]. The roots of *Rhodiola sp.*, one of the important traditional Chinese herbal medicines, have anti-viral [13], anti-tumor [14], anti-ageing, anti-oxygen deficiency, anti-radiation [15] effects and other pharmacological properties [16]. The rhizomes and roots of the Rhodiola plants contain phenylpropanoides: rosavin, rosin, rosarin and their aglycone: cinnamyl alcohol; phenylethanol derivatives: salidroside, tyrosol; flavonoids (catechins and proanthocyanidins), monoterpenes, triterpenes, phenolic acids (gallic, caffeic and chlorogenic acid), volatile oil, amino acids and minerals. Most of these compound posses's antioxidant activity [17, 18]. Considering the importance of this area, present study was focused on some important *in-vitro* evaluation of antioxidant activities and quantification of total phenolic compounds which are responsible for free scavenging activities.

2. Materials and Methods

2.1 Chemicals

Chemical reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), Nitro blue tetrazolium (NBT), Dimethyl sulfoxide (DMSO), Sodium nitroprusside and trichloroacetic acid (TCA) were purchased from Sigma Aldrich Germany, Gallic acid (standard solution), Sodium carbonate and $FeCl_3$ (Merck Chemicals, Mumbai). All other reagents used were of analytical grade.

2.2 Collection of Plant Materials and Extraction

Plant materials were collected in the month of June 2009 from Trans-Himalayan region from Chang-La Top (altitude 17500 ft. Above Mean Sea Level), in India. The collected plant material was identified and authenticated by a scientist Dr. OP Chaurasia, Scientist, Medicinal and Aromatic Plant Division, Defence Institute of High Altitude Research (DRDO), C/o 56 APO, India. A voucher specimen (Specimen no: A –6) has been deposited at the herbarium of our division. The roots were cut into small pieces and shade dried at room temperature for 15 days, finely powdered and air-dried root powder (10 g) was successively extracted by 500 ml methanol with the help of Soxhlet apparatus till the residue remains colourless. The crude extract was concentrated using rotary evaporator under vacuum and reduced pressure at 40 ºC ,which was a viscous dark mass with a percentage yield of 21.4% and the residue was used for further studies.

2.3 Total Polyphenolic Contents

Total polyphenolic were determined by the Folin-Ciocalteau procedure using gallic acid as a standard phenolic compound [19]. Aliquots (0.1 ml) of test-solution were transferred into the test tubes and volumes brought up to 0.5 ml by water. After addition of 0.25 ml Folin-Ciocalteau reagent and 1.25 ml 20% aqueous Na_2CO_3 solution, tubes were vortexed and absorbance of blue-coloured mixtures recorded after 40 min at 725 nm against blank, containing 0.1 ml of extraction solvent. All determinations were performed in triplicate. The amount of total polyphenols was calculated from the calibration curve of gallic acid standard solutions, concentration of total phenols was expressed as mg/g of dry extract.

2.4 DPPH (1, 1 diphenyl 2, picryl hydrazyl) Radical Scavenging Assay

The free radical scavenging activity of the *R. heterodanta* methanol extract was measured *in vitro* by DPPH (1, 1 diphenyl 2, picryl hydrazyl) assay [20]. The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH. 0.1mM solution of DPPH in methanol was prepared and 3.0 ml of this solution was added to 40.0 µl of extract solution in water at different concentrations (.05–0.5 mg/ml). The mixture was incubated at room temperature for 30 minutes and the absorbance was measured at 515 nm against corresponding blank solution. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated based on the control reading using the following equation:

$$\text{DPPH Scavenged (\%)} = (A_0 - A_t) / A_0 \times 100$$

Where A_0 is the absorbance of the control reaction and A_t is the absorbance in the presence of the extract/ standard.
The antioxidant activity of the extract was expressed as IC_{50}, which the concentration (in mg/ml) of extract inhibits formation of DPPH radicals by 50%.

2.5 Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging capacity was measured using modified method as described previously [21]. Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of extract (.05–0.5 mg/ml)each dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37 ^{0}C for 1 h. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of (10%) TCA and 1.0 ml of (0.5%) TBA (in 0.025 M NaOH containing 0.025 M NaOH BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract was reported as the percentage of inhibition of deoxyribose degradation and was calculated according to the following equation:

$$\text{\% inhibition} = (A_0 - A_t) / A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the sample of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values. Ascorbic acid was used as a positive control.

2.6 Nitric Oxide Radical Scavenging Activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide [22] which interacts with oxygen to produce nitric ions that can be estimated by using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate buffer saline (PBS) was mixed with 3.0 ml of different concentrations (.05–0.5 mg/ml) of the RHME and incubated at 25 ^{0}C for 150 min. The samples were added to Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with

sulphanilamide and subsequent coupling with napthylethylenediamine was measured at 546 nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Greiss reagent as a positive control. The percentage of inhibition was measured by the following formula:

$$\% \text{ inhibition} = (A_0 - A_t) / A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract.

All the tests were performed in triplicate and the graph was plotted with the mean values.

2.7 Superoxide Radical-Scavenging Activity

This activity was measured using NBT (Nitro blue tetrazolium reagent) method as described by [23]. The method is based on generation of superoxide radical O^{-2} by auto-oxidation of hydroxylamine hydrochloride in presence of NBT, which gets reduced to nitrite. Nitrite in presence of EDTA gives a color that was measured at 560 nm. Test solutions of extract (.05–0.5 mg/ml) were taken in a test tube. To this, reaction mixture consisting of 1 ml of (50 mM) sodium carbonate, 0.4 ml of (24 mM) NBT and 0.2 ml of 0.1 mM EDTA solutions were added to the test tube and immediate reading was taken at 560 nm. 0.4 ml of (1 mM) of hydroxylamine hydrochloride was added to initiate the reaction then reaction mixture was incubated at 25 ^{0}C for 15 min and reduction of NBT was measured at 560 nm. Ascorbic acid was used as the reference compound. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage of inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = (A_0 - A_t) / A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

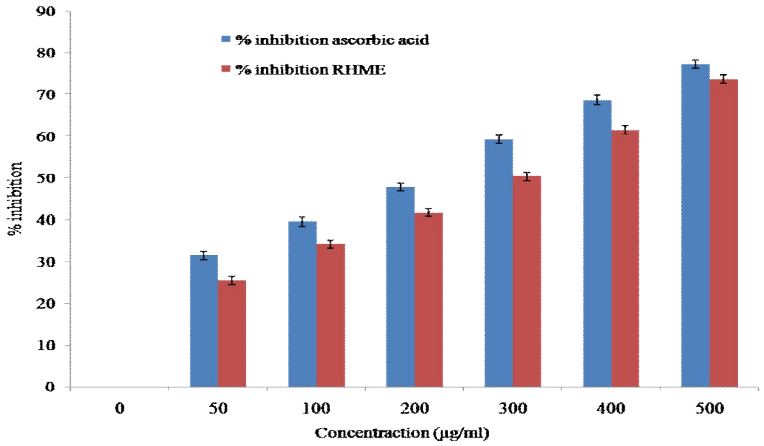

Fig. 1. DPPH radical scavenging activity of the methanolic root extract of *R. heterodanta*
RHME: *Rhodiola heterodanta* methanolic root extract

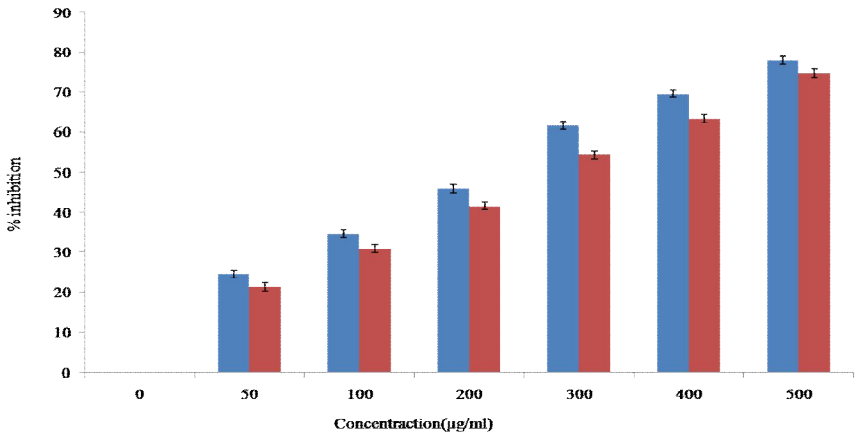

Fig. 2. Hydroxyl radical scavenging activity of the methanolic root extract of *R. heterodanta*
RHME: *Rhodiola heterodanta* methanolic root extract

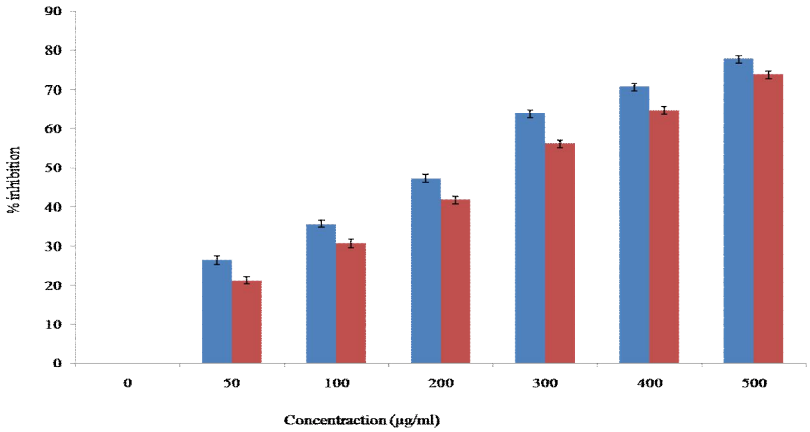

Fig. 3. Nitric oxide radical scavenging activity of the methanolic root extract of *R. heterodanta*
RHME: *Rhodiola heterodanta* methanolic root extract

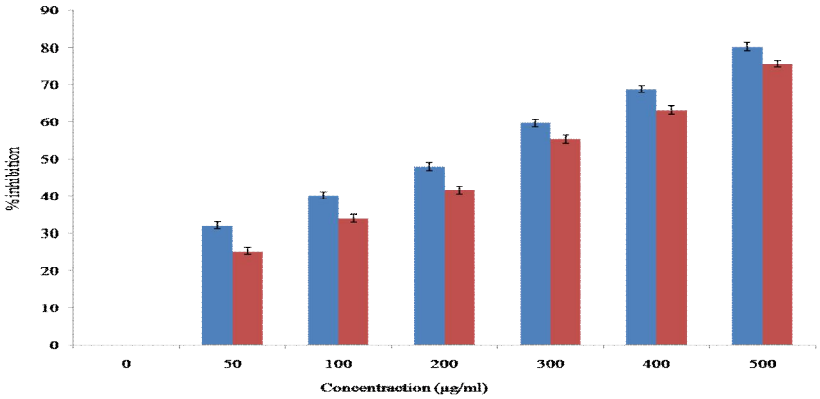

Fig. 4. Superoxide radical scavenging activity of the methanolic root extract of *R. heterodanta*
RHME: *Rhodiola heterodanta* methanolic root extract

3. Result and discussion

3.1 Total Phenolic Content

The content of phenolic compounds (mg/g) in methanol extract of *Rhodiola heterodanta* was found 179.42±.63 mg/g plant extract and expressed in gallic acid equivalents. These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic components. The same relationship was also observed between phenolics and antioxidant activity in rose ship extracts [24]. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups [25]. The phenolic compounds may contribute directly to antioxidative action. It is known that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when ingested up to 1 g daily from a diet rich in fruits and vegetables [26]. Phenolic compounds from plants are known to be good natural antioxidants. However, the activity of synthetic antioxidants was often observed to be higher than that of natural antioxidants [27]. Phenolic compounds, at certain concentrations, markedly slowed down the rate of conjugated diene formation. The interests of phenolics are increasing in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food [28].

3.2 Inhibition of DPPH Radical

The DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals is initiated by the lipid auto-oxidation [29]. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [30]. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 515 nm induced by antioxidants. On the DPPH radical, *R. heterodanta* had significant scavenging effects with increasing concentration in the range of 0.05–0.5 mg/ml when compared with that of ascorbic acid; the scavenging effect of RHME was lower. A 0.5 mg/ml of RHME and ascorbic acid exhibited 73.69% and 77.17% inhibition, respectively (Fig. 1) The IC_{50} values were found to be 0.28 and 0.21 mg/ml for RHME and ascorbic acid, respectively (Table 1). The DPPH activity of RHME was found to increase in dose dependent manner. The RHME at the used concentrations displayed potential effect of DPPH activity as percentage of free radicals inhibition. A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value. These data clearly indicate that RHME is a powerful free radical inhibitor or scavenger.

Table 1: Effect of methanolic root extract of *Rhodiola heterodanta*, on different radical scavenging activities.

IC_{50} value of RHME							
DPPH radical scavenging activity	AA	Hydroxyl radical scavenging activity	AA	Nitric oxide radical scavenging activity	AA	Superoxide radical scavenging activity	AA
0.28	0.21	0.27	0.23	0.28	0.22	0.26	0.19

RHME: *Rhodiola heterodanta* Methanolic Root extract (values in mg/ml).
AA: Ascorbic acid.

3.3 Hydroxyl Radical Scavenging

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells [31]. This radical has the capacity to join nucleotides in DNA and cause strand breakage which contributes to carcinogenesis, mutagenesis and cytotoxicity [32]. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity [33].

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The effect of RHME on the inhibition of free radical-mediated deoxyribose damage was assessed by means of the Fe^{+2}-dependent DNA damage assay. The Fenton reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using Fe^{+2} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. RHME was also capable of reducing DNA damage at all concentrations used. Ascorbic acid was highly effective in inhibiting the oxidative DNA damage.

The % inhibition of RHME (.05–0.5 mg/ml) on hydroxyl radical scavenging was found to be 21.26%, 30.89%, 41.66%, 54.43%, 63.36% and 74.72%, respectively. All results showed antioxidant activity in dose dependent manner. A 0.5 mg/ml of RHME and ascorbic acid exhibited 74.72%and 78.08% inhibition, respectively (Fig. 2) and their respective IC_{50} values were found to be 0.27 and 0.23 mg/ml (Table 1). The ability of the above mentioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of the chain reaction. Ascorbic acid was used as reference standard.

3.4 Nitric Oxide Radical Scavenging Activity

Nitric oxide plays an important role in various types of inflammatory processes in the animal body. In this study, the *R. Heterodanta* methanolic root extract (RHME) was checked for its inhibitory effect on nitric oxide production. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by RHME. The various concentrations of RHME (0.05–0.5 mg/ml) showed 21.26%, 30.66%, 41.7%, 56.11%, 64.7%and 73.89% inhibition, respectively. Results showed the percentage of inhibition in a dose dependent manner (Fig. 3). A 0.5 mg/ml of RHME and ascorbic acid exhibited 73.89% and 77.7% inhibition, respectively. The concentration of RHME needed for 50% inhibition (IC_{50}) was found to be 0.27 mg/ml, whereas 0.22 mg/ml was needed for ascorbic acid (Table 1). These results were found to be statistically significant ($P<0.05$).

3.5 Super Oxide Radical Scavenging

Super oxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases. It was therefore proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the super oxide radical. Several in vitro methods are available for generation of super oxide radicals [34]. In our study super oxide radicals were generated by auto-oxidation of hydroxylamine in presence of NBT (Nitro blue tetrazolium). The reduction of NBT in presence of antioxidants was measured. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The superoxide radical scavenging activity of RHME was almost similar when compared with same doses of ascorbic acid. The various concentrations of RHME (0.05–.5 mg/ml) showed 25.25%, 34.04%, 41.57%, 55.37%, 63.06% and 75.61%inhibition, respectively. Results showed the percentage of inhibition in a dose dependent manner (Fig. 4). A 0.5 mg/ml of RHME and ascorbic acid exhibited 75.61% and 80.25% inhibition, respectively.

All of the extracts had a scavenging activity on the superoxide radicals in a dose dependent manner (0.05-0.5 mg/ml in the reaction mixture). Nonetheless, when compared to ascorbic acid, the superoxide scavenging activity of the extract was found to be low. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract. Results were found statistically significant ($P<0.05$).

4. Conclusion

It is well identified that free radicals are one of the causes of several diseases, such as Parkinson disease, Alzheimer type dementia, etc. The production of free radicals and the activity of the scavenger enzymes against those radicals such as superoxide dismutase (SOD) are correlated with the life expectancies. We have demonstrated the methanolic extract of *R. heterodanta* roots contained high level of total phenolic compounds and were capable of inhibiting, quenching free radicals to terminate the radical chain reaction, and acting as reducing agents. Furthermore, phenolic compounds present in the plant kingdom are mainly responsible for the antioxidant potential of plants. Accordingly in this study, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. The methanolic extract of *R. heterodanta* roots showed strong antioxidant activity by inhibiting DPPH, hydroxyl radical, nitric oxide and superoxide anion scavenging activities when compared with standard ascorbic acid. In addition, the RHME found to contain a noticeable amount of total phenols which plays a major role in controlling antioxidants. These results can be useful for further application of methanolic extract of *R. heterodanta* roots or its constituents in the pharmaceutical preparation after performing clinical *in vivo* research. With this kind of investigation it would be easier to the treat and prevent the human damages occurring due to free radical and also to replace the synthetic antioxidant in industry.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

6. Acknowledgements
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