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In vitro standardization of flowers of methanolic extract of *Dendrobium normale* Falc. for free radical scavenging activity

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Abstract
Traditional remedies, often closely resembling or forming the basis for alternative remedies, may comprise primary healthcare or be integrated into the healthcare system. Numerous diseases are induced by free radicals via lipid peroxidation and protein peroxidation. It has been validated that a variety of herbal extracts have antioxidant activities to scavenge free radicals. *Dendrobium normale Falc.* Belongs to the family *Orchidaceae*; an ethnomedicinal plant. The aim of present work was to evaluate *in vitro* antioxidant potential of methanolic extract of *D. normale* by DPPH, hydroxyl and superoxide radical scavenging assays. The radical scavenging activity was found to be concentration dependent and increased with increased concentrations and produced maximum scavenging activity at a dose of 360 µg. The antioxidant potential could be due to the presence of flavonoids, alkaloids, triterpenoids, steroids and carbohydrates. Further, this can be confirmed by qualitative analysis.

Keywords: antioxidant activity, Dendrobium, flowers, qualitative analysis.

1. Introduction
In developing nations, access to essential medicines is severely restricted by lack of resources and poverty. Many people throughout the world were forced to consider the importance of herbs for treating several forms of diseases and disorders. It is no wonder, during the past decade, there has been an exponential rise in the application of herbal remedies and such notable increase even continues in these days. WHO report 80% of the world population relies on the drugs which are from natural origin [1]. However, several herbal products lining in those shelves are not really standardized in terms of its effectiveness and safety. Experimental evidence suggests that free radicals and reactive oxygen species can be involved in a higher number of diseases via lipid peroxidation, protein peroxidation and DNA damage [2, 3] . It has been known that a variety of plant extracts have antioxidant activities to scavenge free radicals [4]. Pro-oxidant condition dominates either due to the increased generation of the free radicals caused by excessive oxidative stress of the current life, or due to the poor scavenging/quenching in the body caused by depletion of the dietary antioxidants[5]. So it becomes very essential to maintain balance of oxidants in the body. Much attention has been focused on antioxidant compounds present in edible plants, because of safety concerns associated with synthetic antioxidants. Keeping this in mind, the present work was designed and screened the *D. normale* for free radical scavenging activity.

2. Material and Methods
2.1 Selection and collection of plant material
The flowers of *Dendrobium normale* Falc from Talakona forest, Chittoor district of Andhra Pradesh, India and authenticated by Dr. MadhavaChetty, taxonomist and HOD of Botany, Sri Venkateswara University, Thirupathi, India (Voucher specimen No.SVU-Y-99).
Ascorbic acid from Sigma Aldrich Chemie, Germany, Riboflavin from S.D chemicals, India. All other solvents and chemicals used were of analytical grade purchased from local source.

2.2 Preparation of extract
After collection, the flowers were a shade dried, powdered (40 mesh size) to get a coarse powder and then subjected to soxhlet extraction continued for 8 cycles (6 hrs) using methanol as a solvent. The extract was filtered and concentrated at reduced temperature on a rotary evaporator.

The percentage yield was found to be 22.12% w/w and then subjected to preliminary qualitative [6-11] phytochemical analysis [Table 1].

Table 1: Qualitative preliminary phytochemical analysis of methanolic extract of flowers of *Dendrobium normale*

Phytochemical constituents	Methanolic extract
Alkaloids	+
Carbohydrates	+
Phytosterols	+
Saponins	-
Triterpenoids	+
Glycosides	-
Tannins	-
Resins	-
Flavonoids	+

'+' Present '-' Absent

2.3 *In vitro* Free Radical Scavenging Activity

The selected plant methanolic extract was evaluated by DPPH radical scavenging assay, super oxide radical scavenging assay and hydroxyl radical scavenging assay. There is no detailed study on free radical scavenging activity on the selected plant. Hence, a detailed study was carried out.

2.4 Screening for 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

In this method [12], an aliquot of 3 ml of 0.004% DPPH solution in methanol and 0.1 ml of plant extract at various concentrations (40, 80, 120, 160, 200, 240, 280, 320 and 360 µg/ml) were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm. A control was prepared using 0.1 ml of the respective vehicle in the place of plant extract/ascorbic acid. The percentage inhibition activity was calculated by using the following formula [Figure 1].

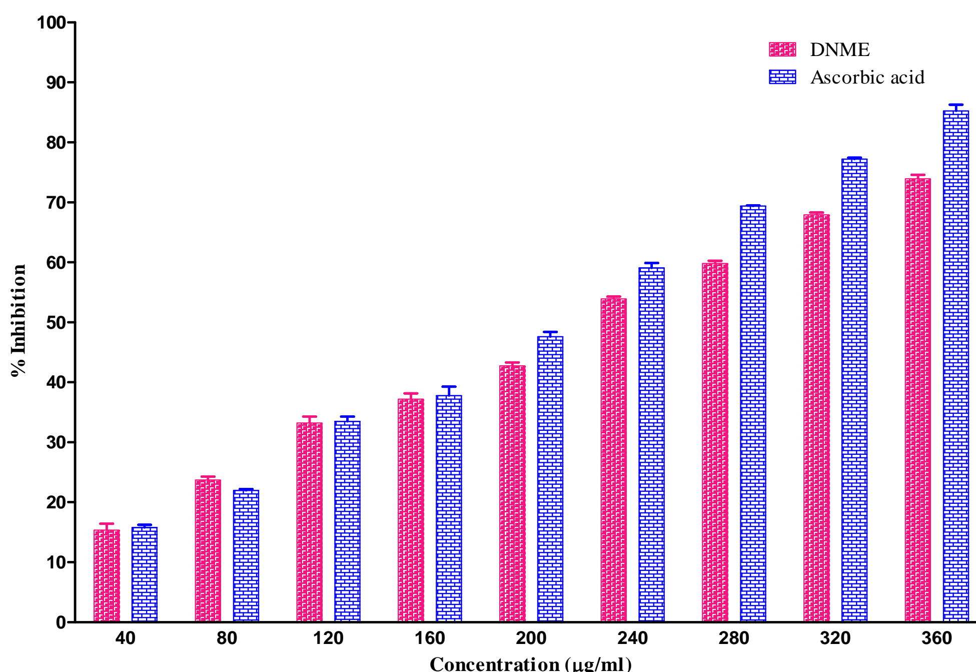


Fig 1: Bar diagram of concentration-dependent percentage inhibition of DPPH radical scavenging activity

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of plant extract/ascorbic acid}}{\text{Absorbance of control}} \times 100$$

2.5 Screening for superoxide radical scavenging activity

Riboflavin photo reduction method [13] was used to screen the selected plant methanolic extract. 0.1 ml of different concentrations (40, 80, 120, 160, 200, 240, 280, 320 and 360 µg/ml) of plant extract and 0.1 ml of 6 µM ethylene diamine tetraacetic acid (EDTA) containing NaCN, 0.1 ml of 50 µM nitroblue tetrazolium, 0.05 ml of 2 µM riboflavin were transferred to a test tube, and final volume was made up to 3 ml using phosphate buffer. Then the assay tubes were uniformly illuminated with an incandescent light (40 Watt) for 15 minutes and thereafter the optical densities were measured at 560 nm. A control was prepared using 0.1 ml of respective vehicle in the place of plant extract/ascorbic acid. The percentage inhibition of superoxide production was evaluated by comparing the absorbance values of control and experimental tubes [Figure 2].

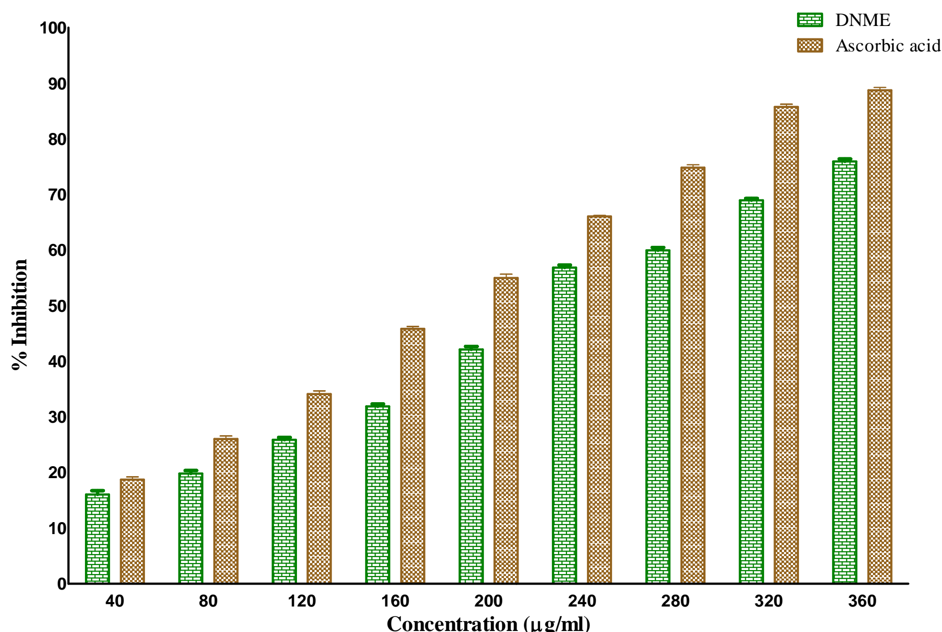


Fig 2: Bar diagram of concentration-dependent percentage inhibition of superoxide radical scavenging activity

$$\%\ \text{Inhibition} = \frac{\text{Absorbance of control - Absorbance of plant extract/ascorbic acid}}{\text{Absorbance of control}} \times 100$$

2.6 Screening for hydroxyl radical scavenging activity

Deoxyribose degradation method [14] was used to screen the different concentrations (40, 80, 120, 160, 200, 240, 280, 320 and 360 µg/ml) of selected plant extract. Fenton reaction mixture consisting of 200 µl of 10 mM ferrous sulphate ($FeSO_4 . 7H_2O$), 200 µl of 10 mM EDTA and 200 µl of 10 mM 2-deoxyribose and was mixed with 1.2 ml of 0.1 M phosphate buffer (pH 7.4) and 200 µl of plant extract. Thereafter, 200 µl of 10 mM H_2O_2 was added before the incubation at 37 ^{o}C for 4 h. Then 1 ml of this Fenton reaction mixture was treated with 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 0.8% thiobarbituric acid and 1.5 ml of 20 % acetic acid. The total volume was then made to 5 ml by adding distilled water and kept in an oil bath at 100 ^{0}C for 1 hour. After the mixture had been cooled, 5 ml of 15:1 v/v butanol-pyridine mixture was added. Following vigorous shaking, the tubes were centrifuged at 4000 rpm for 10 min and the absorbance of the organic layer containing the thiobarbituric acid reactive substances were measured at 532 nm. A control was prepared using 0.1 ml of vehicle in the place of plant extract/ascorbic acid. The percentage inhibition of hydroxyl radicals by the extract/compound was determined by comparing the absorbance values of the control and the experimental tubes as calculated for hydroxyl radical assay [Figure 3].

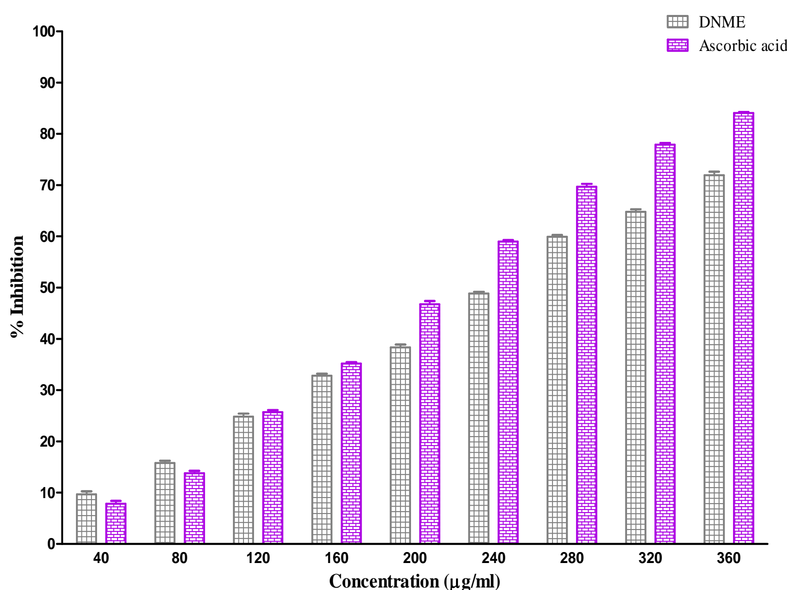
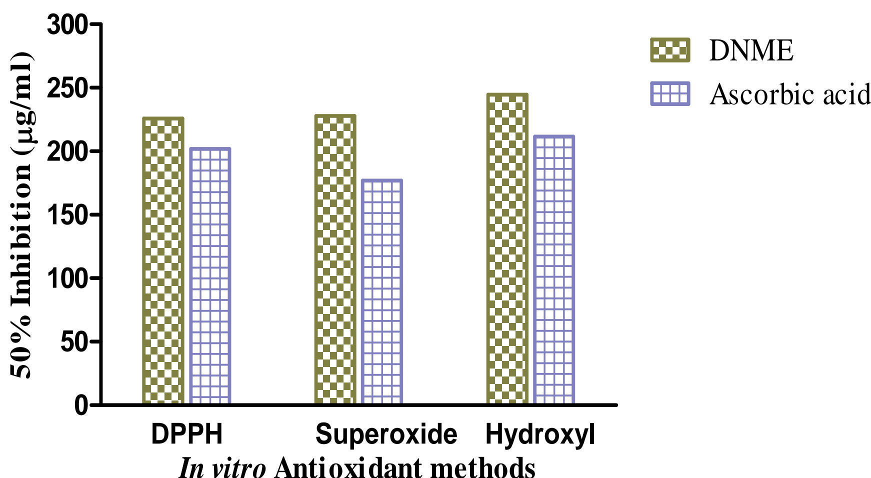


Fig 3: Bar diagram of concentration-dependent percentage inhibition of hydroxyl radical scavenging activity

2.7 Calculation of 50% inhibition concentration
IC_{50} value was calculated for extract and positive control and obtained by plotting a graph by taking concentration on X-axis and %inhibition on Y-axis. The graph was extrapolated to find the concentration needed for 50% inhibition [Figure 4].



DNME: Methanolic extract of *Dendrobium normale*

Fig 4: Bar diagram of IC_{50} values (µg/ml) of methanolic extract of *Dendrobium normale* and ascorbic acid

3. Results and Discussion
Herbal drugs have played an important role throughout the world in treating and preventing variety of human diseases and disorders. The claimed usefulness of medicinal plants in several disorders might be due to their antioxidant potential. To support the use of the selected plant extract in traditional use in Ayurveda and naturopathy, the antioxidant potential of the methanolic extract of flowers of *D. normale* was investigated for free radicals scavenging activity in comparison with the known antioxidant ascorbic acid. The quantities of the extract required for the *in vitro* inhibition of radical such as DPPH, superoxide and hydroxyl were compared to the known antioxidant ascorbic acid.

The DPPH radicals are widely used to investigate the scavenging activity. In the DPPH assay, the antioxidants are able to reduce the stable radical DPPH to the yellow colored diphenyl-picryl hydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. The method helps to determine the antiradical power of an antioxidant by measuring of a decrease in the absorbance of DPPH at 517 nm. Superoxide radical is considered to play an important role in the peroxidation of lipids [15]. At low pH value superoxide will protonate to form the perhydroxyl radical ($HO_2^{\cdot}$), a more reactive oxidizing species, but at physiological pH less than 1%will be in the protonated form [16]. The reactive oxygen radicals are unstable and react readily with other groups or substances in the body, resulting in cell damage and hence human diseases [17]. Among the oxygen radicals specifically, the hydroxyl radical is the most reactive. It severely damages adjacent biomolecules such as all proteins, DNA, PUFA, nucleic acid, and almost any biological molecule it touches. This damage causes ageing, cancer and several diseases. Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases [18, 19].

The selected plant methanolic extract showed dose dependent scavenging activity. The standard drug ascorbic acid also showed similar dose dependent activity and produced maximum scavenging activity at a dose of 360 µg [Figures 1-3]. Qualitative phytochemical studies on the selected plant revealed the presence of flavonoids, alkaloids, triterpenoids, steroids and carbohydrates. The presence of the above said constituents in selected plant extract alone or in combination might be responsible for the observed antioxidant potential.

4. Conclusion
All these scientific observations support the traditional use of *Dendrobium normale* Falc. for treating liver disorders could be due to generation of free radicals. The free radical scavenging and antioxidant properties of phytoconstituents may be the possible mechanism.

5. References

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