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Comparative Study of *In - Vitro* Antioxidant Activity of *Spinacia oleracea* and *Capsicum annuum*

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ABSTRACT

Antioxidants are the vital substances or molecules, which possess the ability to protect or prevent the body from damage caused by free radical, induced oxidative stress. Epidemiological studies specify that intake of fruits and vegetables have the ability to inhibit the damaging behavior of free radicals in the human body. In this study, we assessed the *in - vitro* antioxidative activity of aqueous extracts of commonly used vegetables like *Spinacia oleracea* L. and *Capsicum annuum* L. The extracts were studied for antioxidant activity by Hydroxyl radical scavenging method (Deoxyribose method) and were compared to standard antioxidant Ascorbic acid (AA). The concentration required for 50% inhibition (IC_{50} - Inhibitory Concentration) was calculated. All the extracts showed effective free radical scavenging activity, which increased with increasing concentration. The analysis was made with the use of UV-Vis Spectrophotometer (Model Shimadzu UV-1800) at a wavelength of 532 nm. The present study revealed that Hydroxyl radical scavenging activity of *Capsicum annuum* (IC_{50} value of 2364 µg/ml) was more than that of *Spinacia oleracea* (IC_{50} value of 2474 µg/ml). Many flavonoids and related polyphenols contribute significantly to the total antioxidant activity as well as therapeutic uses of many fruits and vegetables. Natural dietary antioxidants with health benefits like Spinach and Capsicum are preferred because synthetic antioxidants are considered carcinogenic.

INTRODUCTION

Human body has inherent mechanism to reduce the free radical induced injury by endogenous antioxidants. Sometimes these protective mechanisms were found to be not sufficient when compared to the insult produced to the body. Hence, the search for exogenous antioxidants is continued. An antioxidant is a molecule that has the ability to prevent the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. These reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants stop or end these chain reactions by removing free radical intermediates and inhibit other oxidation reactions[1]. They do this by being oxidized themselves, so they are often known as reducing agents. Plants and animals have the capability to maintain complex systems of multiple types of antioxidants such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as *catalase*, *superoxide dismutase* and various *peroxidases*. Insufficient levels of antioxidants or inhibition of the antioxidative enzymes cause oxidative stress and may damage or kill cells. Oxidative stress is damage to cell wall, cell structure, cell function and genetic material by overly reactive oxygen-containing molecules or reactive oxygen species (ROS) [2] like superoxide anion, singlet oxygen, hydroxyl radical and hydrogen peroxide

The oxidative damage initiated by ROS is propagated by lipid peroxidation, which may cause damage to DNA. Oxidative stress seems to play a significant role in many human diseases, including cancers, AIDS, atherosclerosis, stroke, coronary heart disease, diabetes, arthritis, neurodegenerative diseases, liver cirrhosis, aging, altitude sickness etc.[3]

Table 1: Classification of Endogenous Antioxidants [4]

Category	**Examples**
High Molecular Weight Proteins	Albumin, Ceruloplasmin, Transferrin, Haptoglobin.
Low Molecular Weight Proteins	Lipid soluble: Tocopherols, Carotenoids, Quinones, Bilirubin Water soluble: Ascorbic acid, Uric acid.
Enzymes	*Superoxide dismutase, Catalase, Glutathione peroxidase.*

Mechanism of action of Antioxidants [5]

- Electron donation: Primary antioxidants are compounds, which are able to donate hydrogen atom rapidly to a lipid radical, forming a new radical, more stable than the initial one. Biological organs contain many polyunsaturated fatty acids (PUFA) such as linoleic, linolenic and arachidonic acids, mainly in the form of esters of phospholipids, triglycerides or with cholesterol. These PUFA can undergo lipid peroxidation, which can be interrupted by antioxidants by the donation of electrons.

- Metal Chelation: Secondary antioxidants can retard the rate of radical initiation reaction by means of initiators elimination. This can be accomplished by deactivation of high-energy species (e.g. singlet oxygen), absorption of UV light, scavenging of oxygen and thus reducing its concentration, chelation of metal catalyzing free radical reaction or by inhibition of peroxidases such as NADPH oxidase, xanthine oxidase, dopamine-hydroxylase or lipoxygenase.

- Co-antioxidants: Ascorbic acid alone has little effect in preventing lard oil from oxidation. The combination of ascorbic acid with tocopherol gave rise to a strong synergistic antioxidative effect. The role of ascorbic acid was to preserve tocopherol from consumption. This behavior of ascorbic acid is termed as co-antioxidant effect.

- Gene Expression: Antioxidant possesses the ability to donate electrons and thereby act as reducing agents, to chelate metal ions and thereby remove potential radical initiators and to facilitate antioxidant activity by other compounds (co-antioxidants). Antioxidants can also affect directly or indirectly the expression of genes in tissues. A number of genes are regulated by changes in the cellular redox status.

Screening of Antioxidants

Antioxidants are screened by the following methods

- Superoxide radical/Riboflavin Photoreduction method [6].

- Hydroxyl radical/Deoxyribose method.

- Peroxide radical/Induction of Fe^{+2}/ascorbate system method.

Table 2: Natural Sources of Antioxidants [7, 8, 9]

Antioxidant Compound	Sources
Vitamin C	Fresh fruits and vegetables.
Vitamin E	Cereals, nuts, vegetable oils.
Polyphenols (Flavonoids)	Turmeric, tea, coffee, soy, olive oil, chocolate, cinnamon, oregano, apples, grapes, berries.
Carotenoids	Carrot, beetroot, capsicum, tomato, leak, red cabbage, kale
Green leafy vegetables	Spinach, cabbage, mint, parsley, celery, broccoli, peppers, chillies, Brussels sprouts.
Spices	Cloves, pepper, coriander, cumin, dill, fennel, caraway.

Objective of the research [10, 11, 12]

The objective of the research was to determine the antioxidant activity or free radical scavenging activity of commonly used vegetables Spinach (*Spinacia oleracea* L., Family: Amaranthaceae) and Capsicum/Bell pepper (*Capsicum annuum* L., Family: Solanaceae) using *in - vitro* method and comparing the activity with that of a well-established antioxidant Ascorbic acid.

MATERIALS AND METHODS

Spinach and Capsicum were purchased from local market of Hyderabad, India. Ascorbic acid, Ferric chloride and Ethylenediaminetetraacetic acid (EDTA) were purchased from Prime Laboratories, Hyderabad. 2 – Deoxy – D - ribose was a gift sample from SRL Pvt. Ltd, Hyderabad. Disodium phosphate and Sodium dihydrogen phosphate were purchased from S. D. Fine Chemicals, Mumbai, India. Chemicals of analytical grade and deionized water were used throughout the study.

Preparation of Extracts [13]

500 gm. of fresh material of Spinach was washed with water to remove the dirt and later, dried. The dried Spinach was grinded and macerated with 500 ml of water for 1 hour. Later, the mixture was transferred to a round bottomed flask and was boiled for ½ hour attached to a condenser by which the material was never allowed to be dried. After boiling, the mixture was filtered. The filtrate obtained was heated to reduce its volume to half of its original volume and later kept for evaporation by air. In the same way, the extraction of Capsicum was carried out. The crude extracts were re-dissolved in distilled water as and when necessary for the assessment of antioxidant activity.

Preparation of Reagents

- 37.54 mg of 2 – Deoxy – D – ribose was weighed, transferred to a volumetric flask and the volume was made up to 10 ml with distilled water to give 28mM solution. From this 0.1 ml was used in the reaction.

- 37.22 mg of EDTA was weighed, transferred to a volumetric flask and the volume was made up to 100 ml with distilled water to give 1mM solution. From this 0.1 ml was used in the reaction.

- 16.22 mg of Ferric Chloride was weighed, transferred to a volumetric flask and the volume was made up to 100 ml with distilled water to give 1mM solution. From this 0.1 ml was used in the reaction.

- 17.66 mg of Ascorbic acid was weighed, transferred to a volumetric flask and the volume was made up to 100 ml with distilled water to give 1mM solution. From this 0.1 ml was used in the reaction.

- Phosphate buffer solution (PBS), pH7.4:

Solution A: 276 mg of Disodium phosphate ($Na_2HPO_4\ 2H_2O$) was weighed into a beaker and the volume was made up to 100 ml with distilled water.

Solution B: 568 mg of Sodium dihydrogen phosphate ($NaH_2PO_4\ 2H_2O$) was weighed into a beaker and the volume was made up to 100 ml with distilled water.

From the above, 12 ml of solution A and 88 ml of solution B were mixed and the pH was adjusted to 7.4.

Hydroxyl Radical Assay (Deoxyribose Method) [14, 15]

Hydroxyl radical scavenging activity of the extract was found by studying the competition between deoxyribose and the test compounds i.e. extract for the hydroxyl radical generated from Fe^{+3}/ascorbate/EDTA/H_2O_2 system. Hydroxyl radical acts against deoxyribose which results in the formation of thiobarbituric acid reacting substance (TBARS).

Procedure:

Preparation of Test Sample

For the experiment, the extracts of Spinach and Capsicum were used in the concentrations of 3000µg and 6000µg each. To 0.4 ml of phosphate buffer solution (PBS), 0.1 ml of extract solution was added. To the above mixture, 0.1 ml of EDTA, 0.1 ml of $FeCl_3$, 0.1 ml of deoxyribose, 0.1 ml of vitamin C and 0.1 ml of H_2O_2 were added and the reaction mixture was incubated at 37ºC. From the above 1 ml reaction mixture, 0.4 ml was withdrawn and treated with 0.2 ml dodecyl sulphate (8.1 %), 1.5 ml thiobarbituric acid (0.8 %) and 1.5 ml acetic acid (20 %, pH 3.5). The total volume was then made up to 4 ml by adding distilled water and kept in oil bath maintained at 95ºC for 1 hour. After cooling, the absorbance was measured at 532nm (Spectrophotometer Model Shimadzu UV-1800).

The same above experiment was repeated with Ascorbic acid as test sample in different concentrations of 2500µg, 5000µg, 10000µg and 20000µg.

Preparation of Control Sample

Control sample is prepared by the same procedure as that of test sample without the addition of extract / ascorbic acid.

The percentage inhibition was calculated by comparing the optical density of each test sample with that of optical density of control sample by using the below formula

$$\text{Percentage inhibition} = \frac{\text{Control sample O.D - Test sample O.D}}{\text{Control sample O.D}} \times 100$$

The mean and standard error of the mean of five samples of each concentration of Spinach, Capsicum and Ascorbic acid were calculated and presented in Table 3. The data was plotted by taking concentration on X-axis and percentage inhibition on Y-axis as shown in Fig. 1, 2 and 3. From the graph, the concentration required for the 50 % inhibition of hydroxyl radicals was obtained and the results are shown in Table 4. The concentrations of Spinach, Capsicum and Ascorbic acid required for 50 % inhibition were also represented by bar diagram as shown in Fig. 4.

RESULTS AND DISCUSSION

Table 3: Hydroxyl radical scavenging activity of *Spinacia oleracea* and *Capsicum annuum* and Ascorbic acid.

Sr. No.	Extract / Drug	Concentration (µg/ml)	Optical Density	Percentage Inhibition
1.	*Spinacia oleracea*	3000 6000	0.296 0.025	60.63% ± 0.12 96.67% ± 0.47
2.	*Capsicum annuum*	3000 6000	0.275 0.019	63.43% ± 0.31 97.47% ± 0.29
3.	Ascorbic acid	2500 5000 10000 20000	0.502 0.372 0.235 0.160	33.24% ± 0.07 50.53% ± 0.36 68.75% ± 0.51 78.72% ± 0.83

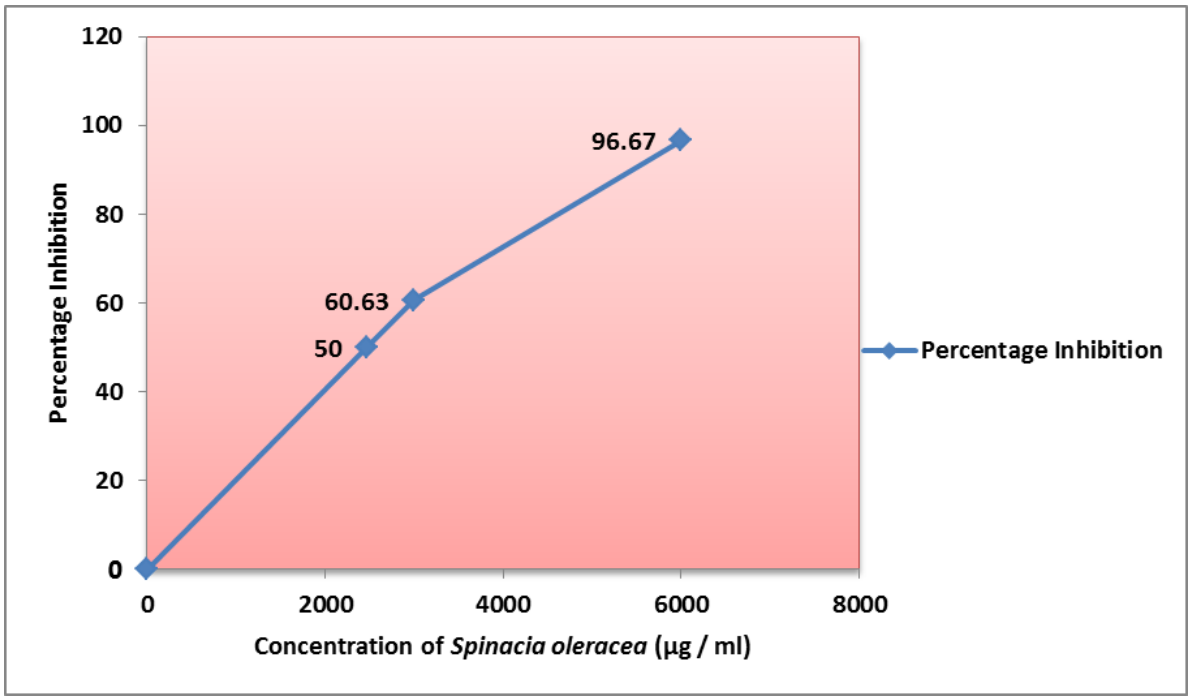

Figure 1: Percentage Inhibition of *Spinacia oleracea*

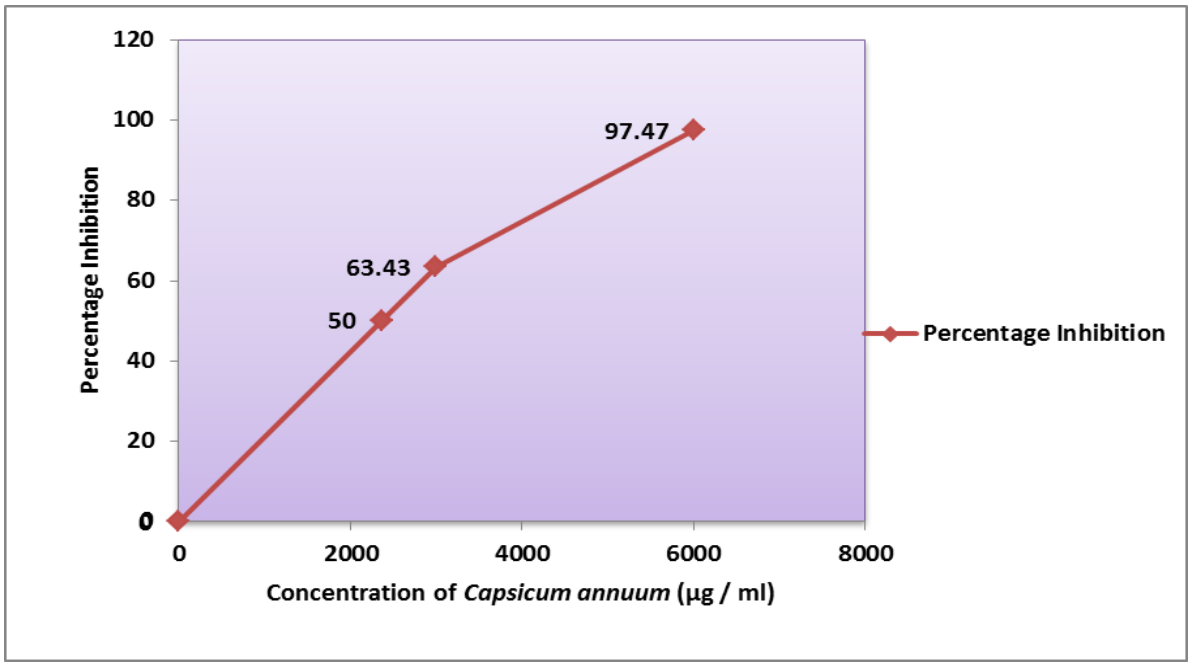

Figure 2: Percentage Inhibition of *Capsicum annuum*

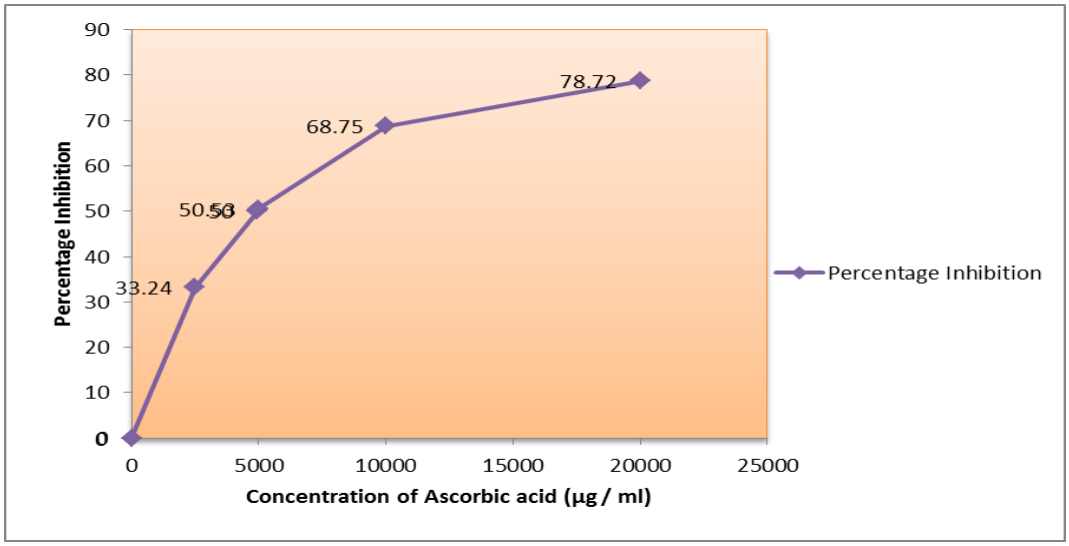

Figure 3: Percentage Inhibition of Ascorbic acid

Table 4: Concentration (µg/ml) at which 50 % of hydroxyl radicals were inhibited

Sr. No.	Extract/Drug	50% Inhibition was obtained at Conc (µg/ml)
1.	*Spinacia oleracea*	2474
2.	*Capsicum annuum*	2364
3.	Ascorbic acid	4937

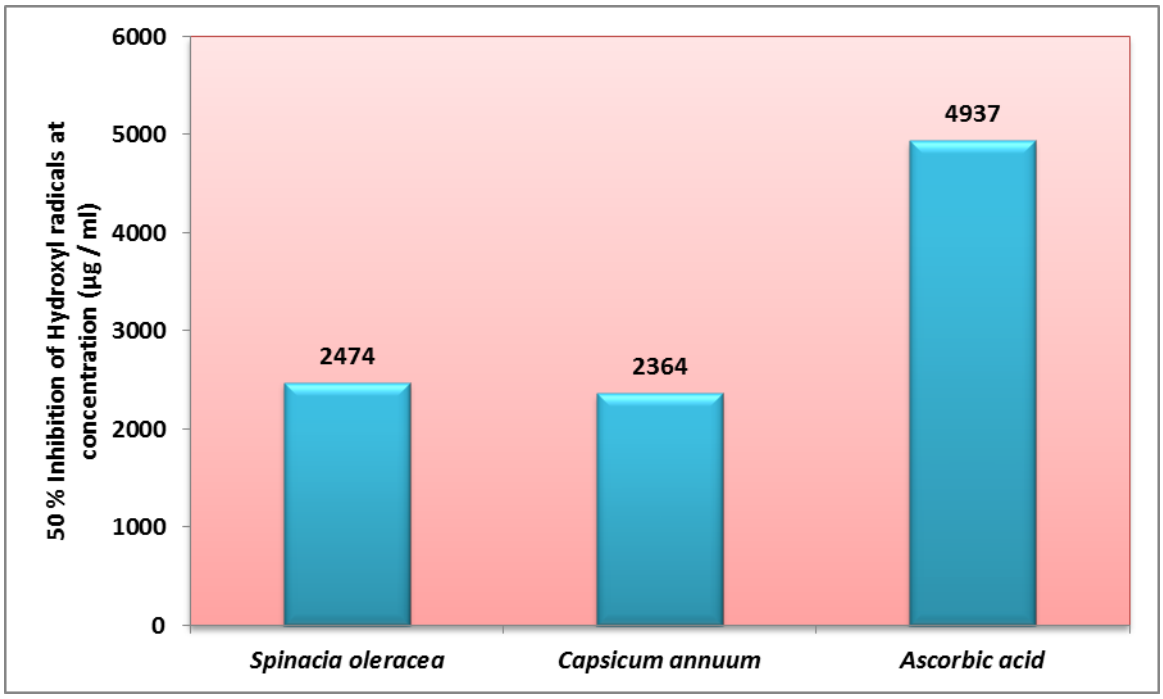

Figure 4: Concentration (µg / ml) required for 50 % Inhibition

Degradation of deoxyribose mediated by hydroxyl radicals generated by Fe^{+3}/ascorbate/EDTA/H_2O_2 system was found to be initiated by both *S. oleracea* and *C. annuum* extracts. The extracts at quantities of 3000µg and 6000µg scavenged the hydroxyl radicals in a dose dependent manner. Ascorbic acid at concentrations of 2500µg, 5000µg, 10000µg, 20000µg was also found to produce dose dependent inhibition of hydroxyl radicals. The quantity of *S. oleracea* extract required to produce 50 % inhibition by hydroxyl radicals was 2474µg whereas that of *C. annuum* extract was 2364µg. Similar effect was produced by Ascorbic acid at concentration of 4937µg.

CONCLUSION

The present study indicated strong antioxidant activity of the aqueous extracts of *Spinacia oleracea*, which was 1.8 – 1.9 times, and *Capsicum annuum*, which was 2 – 2.1 times that of Ascorbic acid, which is a well-established and therapeutically used antioxidant. Since the juice concentrate contains several compounds, it is possible that the antioxidant constituents have been extracted into water. The presence of such compounds might be responsible for their antioxidant activity in *in-vitro* studies and their use in Ayurveda for beneficial effects in certain disorders.

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CONFLICTS OF INTEREST

I have no conflicts of interest to declare.

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