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Research Article

COMPARATIVE STUDIES ON THE IN VITRO ANTIOXIDANT PROPERTIES OF METHANOLIC LEAFY EXTRACTS FROM SIX EDIBLE LEAFY VEGETABLES OF INDIA

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ABSTRACT

Objective: Antioxidants are vital substances which possess the ability to protect 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 antioxidative properties of the methanolic extracts of *Mentha arvensis, Moringa oleifera, Spinacia oleracea, Trigonella foenum-graecum, Tamarindus indica, and Amaranthus viridis*.**Methods:** The methanolic extracts were studied for phytochemical screening and antioxidant properties by different in-vitro experiments including DPPH radical assay, ABTS radical assay, Total antioxidant assay, Reducing activity assay for ascorbic acid equivalents, Total Phenolic content for gallic acid equivalents and Total flavonoid content for quercetin equivalent. **Results:** The present study revealed that *Mentha arvensis* extract exhibited the highest DPPH radical scavenging activity (IC_{50} value of 28 µg/ml), Reducing activity (1.731±0.072), Total antioxidant activity (208 µg/ml expressed as ascorbic acid equivalents), Total phenolic content (75 µg/ml expressed as gallic acid equivalents) and Total flavonoid contents (674 µg/ml expressed as quercetin equivalents) and *Tamarindus indica* extract showed highest ABTS radical scavenging activity (IC_{50} value of 35 µg/ml), The results obtained in the present study indicate that the leaves of *Mentha arvensis* showed potential antioxidant and free radical scavenging activity.**Conclusion:** The results obtained in the present study indicate that leaves of *Mentha arvensis, Moringa oleifera* plant materials have potent, *Trigonella foenum-graecum, Tamarindus indica* have moderate and *Amaranthus viridis, Spinacia oleracea* have mild antioxidant activity and/or free radical scavenging activity.

Keywords: Leafy vegetables, Methanolic extracts, In-vitro antioxidant activity.

INTRODUCTION

Reactive oxygen species (ROS) are constantly formed in the human body by normal metabolic action and these are exert oxidative damaging effects by reacting with nearly every molecule found in living cells including nucleic acids, proteins, lipids or DNA and may involve in several chronic and degenerative diseases including gastritis, reperfusion injury of many tissues, atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others [1,3]**,** If excess ROS and free radicals are not eliminated by endogenous antioxidant system.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Synthetic antioxidant like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and tertiary butyl-hydroquinone (TBHQ) are known to ameliorate oxidative damages but they have been restricted due to their carcinogenic and harmful effect on the lungs and liver [4]. Therefore, investigations of antioxidants are focused on naturally occurring substances, especially plant phytochemicals.

Antioxidant compounds in food play an important role as a health protecting factor. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds such as gallates which has strong antioxidant activity, while others such as the mono-phenols are weak antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals [5].

In view of this screening project, comparative studies on the in vitro activity on methanolic leaf extracts from six indigenous edible leafy vegetables of India were investigated to assess their antioxidant properties in different antioxidant property determination assays include DPPH radical scavenging Method, ABTS radical scavenging activity, Reducing activity assay, Total antioxidant activity, Total Phenol test and Total Flavonoids test were studied in this report. Ascorbic acid, Quercetin and Gallic acid were used as antioxidant standard compounds respectively. The selected leafy vegetables are *Mentha arvensis, Moringa oleifera, Spinacia oleracea, Trigonella foenum-graecum, Tamarindus indica, and Amaranthus viridis*.

MATERIAL AND METHODS

Chemicals and Reagents

Chemicals such as 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid (ABTS) were procured from Sigma Chemical Co. (INDIA). All other chemicals unless and otherwise mentioned were obtained from Hi-media Laboratories and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India).

Spectrophotometric Measurements

Spectrophotometric measurements were performed by UV-VIS Double Beam Spectrophotometer **(ELICO SL-210).**

Plant material

Total six leafy vegetables were purchased from local market, YSR District, Andhra Pradesh, India in the month of January 2011. The plants were authenticated by Dr. A. Madhusudhana Reddy, Taxonomist, Department of Botany, Yogi Vemana University, Kadapa District, Andhra Pradesh, India. Voucher specimens of the collected plants were deposited in the laboratory of Phytochemistry and Pharmacognosy, Fathima Institute of Pharmacy, YSR District, Andhra Pradesh, India for future reference. The leafy vegetables were

cleaned and shade-dried for a week, powdered mechanically (sieve no: 10/44) and stored in air tight containers.

Preparation of extracts

The fresh whole leafy vegetables were chopped and dried in shade. The dried masses were blended into fine powder by frequent sieving and powders were extracted by soxhlet process with methanol. After extraction the contents were concentrated at maintained proper conditions and dried in desiccators to get corresponding extracts. All the extracts were stored at 0^0c in airtight containers until need for further studies.

Preliminary phytochemical investigation

The preliminary phytochemical screening of the extracts was carried out to know the different constituents present in extracts as per the standard procedures [6] and mentioned in table 1.

Determination of Antioxidant capacity

DPPH Radical Scavenging Activity

10 ml of the different concentrations of leaf extract/standard was centrifuged at 3000 rpm using a centrifuge for 10 minutes and supernatant collected. The supernatant of the extract (1 ml) was added to 3 ml of methanolic solution of DPPH (20 mg/l) in a test tube. The reaction mixture was kept at 25^0C for one hour in an incubator .The absorbance of the residual DPPH solution was determined at 517 nm in a UV-Visible Spectrophotometer. The experiment was performed in triplicate. Vitamin C was used as positive control [7].The inhibition was calculated in following formula,

$$I\,(\%) = 100 \times (A_0-A_1)/A_0$$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the extract/standard, respectively. A percent inhibition versus concentration curve was plotted and the concentration of sample required for % 50 inhibition was determined and expressed as IC_{50} value. The lower the IC_{50} value indicates high antioxidant capacity.

ABTS radical scavenging Activity

The ABTS radical cation preparation: ABTS 2 mM (0.0548 gm in 50 ml) was prepared in distilled water. Potassium per sulphate 70 mM (0.0189g in 1ml) was prepared in distilled water. 200 µl of potassium persulphate and 50 ml of ABTS were mixed and used after 2 hrs. This solution was used for the assay. To the 0.5 ml of various concentrations of methanolic extract/standard, 0.3 ml of ABTS radical cation and 1.7 ml of phosphate buffer pH 7.4 was added and the absorbance was measured at 734 nm. Vitamin C was used as positive control [8,9]. The experiment was performed in triplicate. The inhibition was calculated in following way:

$$I\,(\%) = 100 \times (A_0-A_1)/A_0$$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the extract/standard. A percent inhibition versus concentration curve was plotted and the concentration of sample required for % 50 inhibition was determined and expressed as IC_{50} value. The lower the IC_{50} value indicates high antioxidant capacity.

Total antioxidant activity

The total antioxidant activity was eluted by using the method described by Prieto et al (1999). Plant extracts were dissolved in methanol to obtain a concentration of 500 µg/ml. 3 ml of extract was placed in a test tube, 0.3 ml of reagent solution (0.6 M Sulphuric Acid, 28 mM Sodium Phosphate, 4 mM Ammonium molybdate) was then added and the resulting mixture was incubated at 95^0C for 90 minutes. After the mixture was cooled to room temperature, the absorbance of the each solution was measured by using UV-Visible spectrophotometer at 695 nm against blank. The experiment was performed in triplicate [7]. A calibration curve was constructed, using ascorbic acid (100-500 µg/ml) as standard and total antioxidant activity of extract (µg/ml) expressed as ascorbic acid equivalents.

Total phenolic content

Total phenolic content were quantified and expressed as Gallic acid equivalents according to a method proposed by Singleton et al., (1999). Plant extracts were dissolved in methanol to obtain a concentration of 500 µg/ml. About 3.9 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent were added to 0.1 ml of the extract in a tube and incubated at room temperature for 3 minutes, 2 ml of 20 % sodium carbonate was added to this and kept on boiling water bath for minute. The blue colour formed was read at 650 nm. The experiment was performed in triplicate [10]. A calibration curve was constructed, using gallic acid (100-500 µg/ml) as standard and total phenolic content of the extract (µg/ml) expressed as gallic acid equivalents.

Total flavonoid content

Plant extracts were dissolved in methanol to obtain a concentration of 500µg/ml. 1 ml of test sample and 4 ml of distilled water were added to a volumetric flask (10 ml capacity) and kept for five minutes, then adding 0.3 ml of 5% NaNO2, 0.3 ml of 10% $AlCl_3.H_2O$ were added. After six minutes of incubation at room temperature, 2 ml of 1M NaOH was added to the reaction mixture. Immediately the final volume was made up to 10 ml of distilled water. The absorbance was measured at 510 nm. The experiment was performed in triplicate [10]. A calibration curve was constructed, using quercetin (100-500 µg/ml) as standard and total flavonoid content of the extract (µg/ml) expressed as quercetin equivalents.

Reducing Power Activity

2.5 ml of different concentrations of extract/standard was mixed with phosphate buffer (2.5 ml, 0.2 M, P^H 6.6) and potassium ferricyanide (2.5 ml, 1%). This was incubated at 50°C for 20 min. After the incubation, 2.5 ml of 10% trichloroacetic acid was added. 2.5 ml of the reaction mixture was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%). The solution absorbance was measured at 700 nm. The experiment was performed in triplicate. Vitamin C was used as positive control. Increase in absorbance of the reaction mixture indicated the increased reducing power of the samples [11].

Statistical Analysis

The experimental results were expressed as mean±standard deviation (SD) of three replicates.

RESULTS AND DISCUSSION

Phytochemical Screening

Trigonella foenum-graecum and *Amaranthus viridis* shows alkaloids, Glycosides and Steroids positive, *Moringa oleifera* and *Tamarindus indica* shows alkaloids and glycosides positive, *Spinacia oleracea* and *Mentha arvensis* shows alkaloids, glycosides, steroids, flavonoids and phenolic compounds positive.

Table 1: Preliminary Phytochemical Screening of Leafy Vegetable Extracts

Test Name	Trigonella Foenum- Graecum	Moringa Oleifera	Tamarindus Indica	Amaranthus Viridus	Spinacia Oleracea	Mentha Arvensis
Alkaloids	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Steroids	+	-	-	+	+	+
Flavonoids	-	-	-	-	+	+
Phenolic compounds	-	-	-	-	+	+

DPPH Radical Scavenging Activity

The stable DPPH radical model is a widely used, relatively quick and precise method for the evaluation of free radical scavenging activity. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH both transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and convert it to 1-1,diphenyl-2- picryl hydrazine and the degree of discoloration indicates the scavenging activity of the drug. The reduction capacity of DPPH radical is determine by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants because of the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in color from purple to yellow. Hence DPPH is usually used as a substance to evaluate the antioxidant activity [12,13]. Table: 2 shows DPPH radical scavenging activity of standard ascorbic acid. The extract of *Mentha arvensis* was showed highest DPPH scavenging activity and compared with ascorbic acid as standard IC_{50} value is 6.8 µg/ml and result IC_{50} value is 25 µg/ml. Antioxidant activity of leafy green extracts were showed in following order: Ascorbic acid (6.8 µg/ml) > *Mentha arvensis* (25 µg/ml) > *Moringa oleifera* (185 µg/ml) > *Amaranthus viridis* (190 µg/ml) > *Tamarindus indica* (210 µg/ml) > *Spinacia oleracea* (475 µg/ml) > *Trigonella foenum-graecum* (620 µg/ml).

Table 2: DPPH Radical Scavenging Activity

S. No	Name of the leafy vegetable extracts	IC_{50} Value
1	*Mentha arvensis*	25 µg/ml
2	*Moringa oleifera*	185 µg/ml
3	*Spinacia oleracea*	475 µg/ml
4	*Trigonella foenum-graecum*	620 µg/ml
5	*Tamarindus indica*	210 µg/ml
6	*Amaranthus viridis*	190 µg/ml
7	Ascorbic Acid	6.8 µg/ml

ABTS radical scavenging Activity

ABTS is also frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods. In this assay, ABTS is converted to its radical cation by addition of potassium persulfate. This radical cation is blue in color and absorbs light at 734 nm. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and ascorbic acid. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form. Table: 3 shows ABTS radical scavenging activity of standard ascorbic acid. The extract of *Tamarindus indica* was showed highest ABTS scavenging activity and compared with ascorbic acid as standard IC_{50} value is 13.7 µg/ml and result IC_{50} value is 35 µg/ml. Antioxidant activity of leafy green extracts were showed in following order: Ascorbic acid (13.7 µg/ml) > *Tamarindus indica* (35 µg/ml) > *Mentha arvensis* (40 µg/ml) > *Spinacia oleracea* (180 µg/ml) > *Trigonella foenum-graecum* (190 µg/ml) > *Moringa oleifera* (300 µg/ml) > *Amaranthus viridis* (500 µg/ml).

Table 3: ABTS Radical Scavenging Activity

S. No	Name of the leafy vegetable extracts	IC_{50} Value
1.	*Mentha arvensis*	40 µg/ml
2.	*Moringa oleifera*	300 µg/ml
3.	*Spinacia oleracea*	180 µg/ml
4.	*Trigonella foenum-graecum*	190 µg/ml
5.	*Tamarindus indica*	35 µg/ml
6.	*Amaranthus viridis*	500 µg/ml
7.	Ascorbic Acid	13.7 µg/ml

Total antioxidant activity

The Phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds [3]. Figure: 1 shows total antioxidant activity of standard ascorbic acid. The extract of *Mentha arvensis* showed highest total antioxidant capacity and it was 215 µg/ml calculated as Ascorbic acid equivalents was detected. The total antioxidant activity of methanolic extracts exhibited the following order: *Mentha arvensis* (215 µg/ml) > *Moringa oleifera* (93 µg/ml) > *Trigonella foenum-graecum* (78 µg/ml) > *Tamarindus indica* (72 µg/ml) > *Amaranthus viridis* (70 µg/ml) > *Spinacia oleracea* (66 µg/ml).

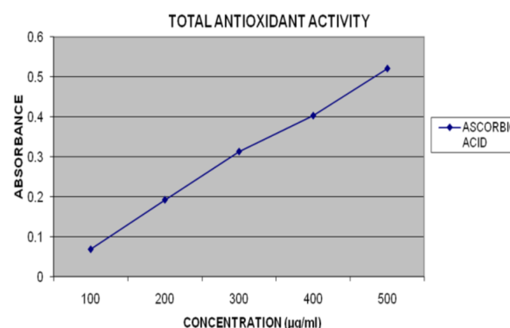

Figure 1: Total antioxidant activity

Total phenolic content

Many plant extracts have been reported to have multiple biological effects, including antioxidant properties due to their phytoconstituents including phenolics. The antioxidant activity of phenolics is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [5]. Figure: 2 shows total phenolic content of standard Gallic acid. The extract of *Mentha arvensis* showed highest total phenolic content and it was 75 µg/ml calculated as Gallic acid equivalent of phenols was detected. The total phenolic content of methanolic extracts exhibited the following order: *Mentha arvensis* (75 µg/ml) > *Moringa oleifera* (60 µg/ml) > *Spinacia oleracea* (20 µg/ml) > *Trigonella foenum-graecum* (12 µg/ml) > *Tamarindus indica* (10 µg/ml) > *Amaranthus viridis* (5 µg/ml).

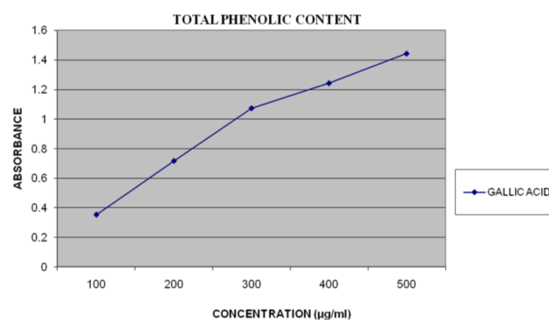

Figure 2: Total Phenol content

Total flavonoid content

Flavonoids comprise the most widespread and diverse group of polyphenolic plant secondary metabolites. These compounds play an important role in biological activities includes antibacterial, antiviral, and anti-inflammatory, anti allergic, antithrombotic, vasodilatory actions and also exhibit free radical scavenging properties by either through scavenging or chelating process [13,14,15]. Figure: 03 shows total flavonoids content of standard Quercetin. The extract of *Mentha arvensis* showed highest total phenolic content and it was 675 µg/ml calculated as Quercetin equivalent flavonoids was detected. The total flavonoids content of methanolic extracts exhibited the following order: *Mentha arvensis* (675 µg/ml) > *Moringa oleifera* (415 µg/ml) > *Tamarindus indica* (205 µg/ml) > *Trigonella foenum-graecum* (190 µg/ml) > *Amaranthus viridis* (155 µg/ml) > *Spinacia oleracea* (125 µg/ml).

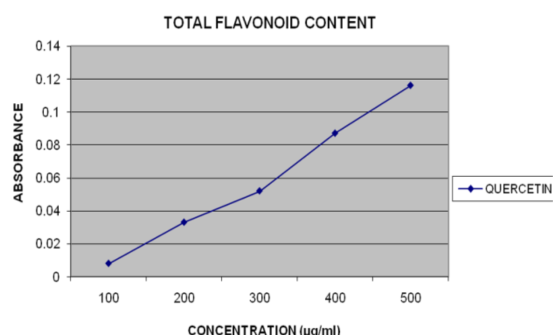

Figure 3: Total flavonoid content

Reducing Power Activity

For the measurements of the reductive ability it has been investigated from the Fe^{3+} to Fe^{2+} transformation in the presence of extract samples using the method described by 0yaizu. Figure: 04 shows reducing power of methanolic extracts and standard ascorbic acid. The reducing power of methanolic extracts and standard compound ascorbic acid exhibited the following order: Ascorbic acid > *Mentha arvensis* > *Moringa oleifera* > *Spinacia oleracea* > *Trigonella foenum-graecum* > *Tamarindus indica* > *Amaranthus viridis*. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain or by donating a hydrogen atom [3].

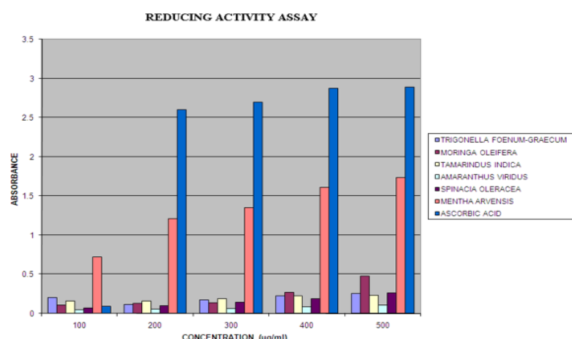

Figure 4: Reducing activity Assay

CONCLUSION

In conclusion, the results of the present study suggest that Mentha arvensis, Moringa oleifera plant materials have potent, Trigonella foenum-graecum, Tamarindus indica have moderate and Amaranthus viridus, Spinacia oleracea have mild antioxidant activity and/or free radical scavenging activity. The providing data can enrich the existing comprehensive data of free radical scavenging activity of plant materials.

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