ESTIMATION OF ANTIOXIDANT PROPERTY OF NATURAL LEAF EXTRACTS BY *INVITRO* METHODS: A COMPARITIVE STUDY

M. MANOJ KUMAR*, V. AKHILA

M. MANOJ KUMAR, ASSOCIATE PROFESSOR, DEPARTMNT OF PHARMACOLOGY, SIR C. R. REDDY COLLEGE OF PHARMACEUTIVAL SCIENCES, ELURU, WEST GODAVARI DISTRICT, ANDHRA PRADESH, INDIA.

V. AKHILA, DEPARTMNT OF PHARMACOLOGY, SIR C. R. REDDY COLLEGE OF PHARMACEUTIVAL SCIENCES, ELURU, WEST GODAVARI DISTRICT, ANDHRA PRADESH, INDIA.

ABSTRACT

Health is the main issue of today's life. Most of the diseases are due to oxidative stress. Oxidation usually causes lysis of the cells. Plants such as *Moringa oleifera, Spinacia oleracea, Murraya koenigii, Piper betel, Mentha piperita* are commonly used in Indian kitchen. They are also used medicinally for the management of different human diseases. The Antioxidant activities of leaves from these herbs were investigated using hydro alcoholic extracts.

The extracts are subjected to phytochemical analysis [1]. *Moringa oleifera* was found to contain alkaloids, saponins, flavanoids, tannins. *Spinacia oleracea* was found to contain alkaloids, saponins, flavanoids, glycosides. *Murraya koenigii* contains alkaloids, saponins, flavanoids, terpenoids and fats. *Piper betel* contains alkaloids, saponins, steroids. *Mentha piperita* was found to contain alkaloids, saponins, flavanoids, tannins, terpenoids, glycosides, fats.

The three different *invitro* methods were used namely reducing power assay[2], Hydrogen peroxide scavenging activity[3], Catalase enzyme activity [4]using the standard antioxidant drug (Ascorbic acid) for the estimation of antioxidant activity. All the five natural leafy extracts showed antioxidant activity. But *Spinacia oleracea* has shown highest activity. Results are almost comparable with standard drug ascorbic acid in dose 60 µg. Hence the leaves of these herbs are useful as antioxidants.

Key Words

Natural Anrioxidants, oxidative stress, Antioxidants in kitchen, Antioxidant food suppliments

INTRODUCTION

Nature is the phenomenone of physical world collectivity including plants, animals, the landscape and the other features and products of earth as opposed to humans and human creations. Nature is a close associate to the life. It is treasure to human beings. It provides every thing to human beings including remedies for different ailments. The only task of humans is to identify them. One such remedy is provided by nature to humans is natural antioxidant which fight against oxidative stress. There are many natural antioxidant substances available in nature. Our work is focused to identify some of such antioxidant substances.

Antioxidants are natural and synthetic substances that can prevent cell damage caused by free radicals, unstable molecules that the body produces as a reaction to environment and other pressures[5]. During aerobic respiration free radicals are generated in humans, generated free radicals in humans, plants and animals are deactivated by antioxidants. Free radicals may be produced by physiological or biochemical process which are capable of reacting with membrane lipids, enzymes, proteins nucleic acid and some molecules leading to cellular damage.

"Antioxidants are acting as Free Radicals Scavenger's" thereby protecting body. Free radicals are short half life, highly reactive, damaging activity towards macro molecules such as DNA, Proteins and Lipids. Oxidative damage to DNA causes premature aging and Wrinkles [6], it is important to protect our skin as it is largest organ of human body. Antioxidants reduce the Free Radicals thereby limiting the signs of aging, wrinkles, inflammation, scars, sun damage.

Naturally occuring antioxidants includes flavanoids, saponins, Fresh fruits, vegetables, whole grains are rich in phytochemicals with antioxidant properties alkaloids, tannins, phenols and liganes[7]. Green leaves are most abundant source of proteins, vitamins and minerals. Plants are rich in antioxidants for the Free Radical scavenging such as vitamins, phenolic acids, terpenoids, alkaloids, quinones, flavanoids, tannins, liganas, stilbenes, amines, coumarins, betalains and some other metabolites.. So, experts believe that antioxidants from food are most healthful way.

Oxygen essential for life of aerobic organism, in higher amounts it may be toxic. Consuming excess amount of antioxidants may be harmful. For Example Vitamin E supplements may increase the risk of bleeding who are receiving anticoagulant drugs[8], high dosage of beta-carotene supplements increased risk of lung cancer in smokers. The main common antioxidants are beta-carotene, lycopen and some of vitamins such as Vitamin A, Vitamin C and Vitamin E.

MATERIALS AND METHODS

Plant Collection

Collection of Leaves from these herbs such as *Moringa Oleifera, Spinacia Oleracea, Murraya koenigii, Piper Betel, Mentha Piperita* washed thoroughly under running tap water and sliced into small pieces and then shade dried until it gives smooth powder of grinding[9].

Preparation of Extract

Above powder is extracted with hydro-alcoholic solution, i.e., Methanol and water in 1:1 ratio[10]. This process is continued for two weeks to result in complete extraction. Then the above solution is evaporated for the extract, collect the extract and store for further use.

Qualitative phytochemical analysis of aqueous extracts of the powdered medicinal crud drugs

Detection of bioactive components present in the medicinal plants by phytochemical screening which is a valuable step for developing the drug discovery[11].

Table-1 Phytochemical analysis

Phytoconstituents	MK EXTRACT	PB EXTRACT	MO EXTRACT	SO EXTRACT	MP EXTRACT
Alkaloids	Present	Present	Present	Present	Present
Saponins	Present	Present	Present	Present	Present
Glycosides	Absent	Absent	Absent	Present	Present
Carbohydrates	Absent	Absent	Absent	Absent	Absent
Tannins	Absent	Absent	Present	Absent	Present
Flavonoids	Present	Present	Present	Present	Present
Steroids	Absent	Absent	Absent	Absent	Absent
Proteins	Absent	Absent	Absent	Absent	Absent
Terpenoids	Present	Present	Present	Absent	Present

Materials required for antioxidant activity

Standard drug : Ascorbic acid

Test drug : Natural extracts

Chemicals required : 1% potassium Ferric cyanide, 10% Trichloro acetic acid, Distilled water, 0.1% Ferric chloride, hydrogen peroxide, plasma, ammonium chloride, sodium bicarbonate, ethylene diamine tetra acetic acid, phosphate buffer.

Preparation of phosphate buffer (pH 7.0):

- 0.5gm anhydrous disodium hydrogen phosphate and 0.301gm of potassium dihydrogen phosphate is dissolved in 1000ml water[12].

Preparation of phosphate buffer (pH 6.6):

- 0.2 M Monobasic stock :

13.90 g of sodium phosphate mono basic di hydrate in 500 ml distilled water.

- 0.2 M Dibasic stock :

Dissolve 26.825 g of sodium phosphate dibasic hepta hydrate in 500ml distilled water. From sample A take 375 ml and mix 225 ml of sample B and make up to 1000 ml[12].

Preparation of phosphate buffer (pH 7.4):

- 0.1M Stock Preparation:

10.9gm of disodium hydrogen phosphate and 3.1gm of sodium dihydrogen phosphate are dissolved in 1000ml of distilled water[12].

Preparation of lysate solution:

Ammonium Chloride (NH4Cl) – 8.02gm

Sodium bicarbonate (NaHCo3) – 0.84gm

EDTA (disodium) – 0.37gm

Ammonium chloride, Sodium bicarbonate, EDTA (disodium) is dissolved in some amount of water and final volume is make up to 100ml with water and it can be stored at 4◦C for 6 months (stock solution).

For working standard solution: From the above stock solution 10ml is taken and volume is make up to 100 ml with water (1:10) (refrigerate the solution until use).[13][14]

Methods used for determining antioxidant activity

1. Reducing power assay
2. Hydrogen peroxide scavenging activity
3. Catalase enzyme activity

Reducing power assay method

The reducing power was determined according to method described by Oyaizu (1986)[12]. Various concentrations of natural extracts (100, 200, 300, 400, 500, 600µg/ml)in corresponding solvents were mixed with 2.5ml of sodium phosphate bufferat pH 6.6 and 2.5ml of 1%potassium ferricyanide. This mixture was incubated at 50ºC for 20 minutes, after cooling 2.5ml of 10%trichloroacetic acid and centrifuged at 800rpm if necessary. The upper layer of 2.5ml of solution was mixed with 2.5 ml of distilled water and0.1ml of freshly prepared 0.1%ferric chloride solution was added. Control was prepared in the similar manner excluding samples; ascorbic acid at various concentrations such as 50, 100, 150, 200, 250, 300µg/ml was used as standard. The absorbance was measured at 670nm. Increase absorbance of reaction mixture indicates increase in reducing power.

Hydrogen peroxide scavenging activity

The ability of natural extracts to scavenge hydrogen peroxide was determined according to the method described by Ruch et al (1989)[13].

A solution of 40mM hydrogen peroxide was prepared in sodium phosphate buffer at pH 7.4. Various concentrations of natural extracts such as 50,100,150,200,250,300µg/ml were added to 3ml of 40Mm hydrogen peroxide, then the final volume was made upto 10ml. Control was prepared in the same manner excluding samples, ascorbic acid at various concentrations 10, 20, 30, 40, 50, 60µg/ml corresponding to the natural extracts. Absorbance of hydrogen peroxide at 230nm was measured after 10 minutes.

Catalase enzyme activity

Catalyse activity can be determined in erythrocyte lysate by Samuel [14].

200µg/ml plasma is added to 3ml of lysate solution, to that add 0.1ml drug at various concentrations50,100,150,200,250,300µg/ml, 2ml of phosphate buffer at pH 7.0 and 1ml of 30mM hydrogen peroxide then make upto 10ml.control was prepared in the same make excluding samples, ascorbic acid was taken as standard at various concentrations such as 10,20,30,40,50,60µg/ml. Catalyse activity is measured at 240 nanometre for 1min using spectrophotometer.

RESULTS

Reducing power assay method

All the natural extracts at various concentrations 100, 200, 300, 400, 500, 600µg/ml are compared with standard drug ascorbic acid. The absorbance represents absorbance of Iron in ferrous form. Increase in acsorbance indicates increase in reducing power. The maximum absorbance in this study is produced by the 600µg dose of the standard drug, Ascorbic acid. Considering that as 100%, reducing powers respective to all other absorbamces are calculated.

Table-2 Data for Reducing power assay method

Sno	Conc	Ascorbic acid		Murayya koenigii		Piper Betel		Moringa oleifera		Spinacia oleracea		Mentha piperita	
		Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd
1	100	0.149	18	0.091	11	0.091	11	0.111	13	0.171	20	0.131	15
2	200	0.337	40	0.182	22	0.172	20	0.182	21	0.313	37	0.181	21
3	300	0.466	55	0.233	28	0.233	28	0.253	30	0.424	50	0.302	36
4	400	0.564	67	0.314	37	0.273	32	0.313	37	0.514	61	0.362	43
5	500	0.709	84	0.374	44	0.338	40	0.394	47	0.605	72	0.413	49
6	600	0.844	100	0.445	53	0.435	51	0.497	59	0.699	83	0.444	53

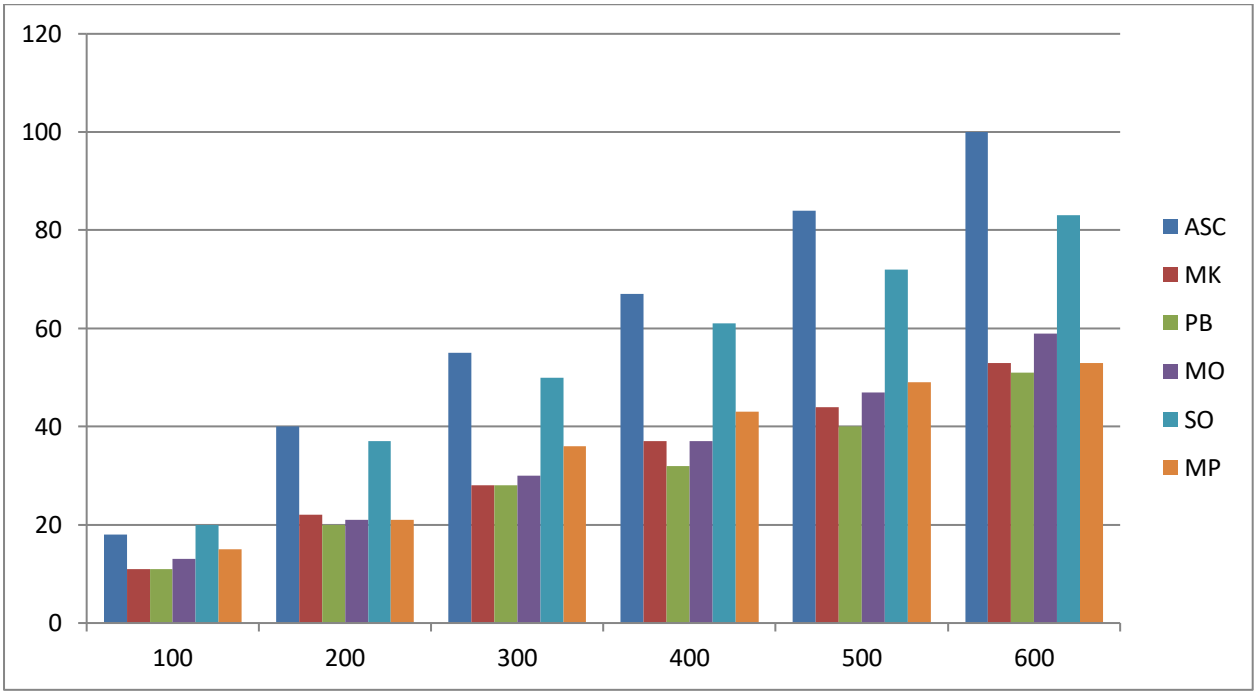

Figure-1 Reducing power assay method

In the graph AC-Ascorbic acid, MO- *Moringa olefera*, MK- *Murraya koenigii*, MP- *Mentha piperita*, SO- *Spinacia oleracea*, PB- *Piper betel.*

Hydrogen peroxide scavenging activity

All the natural extracts at various concentrations 50, 100, 150, 200, 250, 300µg/ml are comparedMwith standard drug ascorbic acid. The control absorbance here is initial absorbance of hydrogen peroxide in the absence of any antioxidant drug. Decrease in absorbance after addition of drug indicates decrease in concentration of hydrogen peroxide which is an indication of reduction of hydrogen peroxide. The reduction of hydrogen peroxide is nothing but antioxidant activity. Decrease in absorbance from the control is used for calculation of % Reduction of hydrogen peroxide. This indicates antioxidant effect of the drug.

Table-3 Data for Hydrogen peroxide scavenging activity Control absorbance -0.542.

Sno	conc	Ascorbic acid		Murayya koenigii		Piper betel		Moringa oleifera		Spinacia oleracea		Mentha piperita	
		Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd
1	50	0.428	21	0.466	14	0.455	16	0.434	20	0.385	29	0.472	13
2	100	0.321	41	0.428	21	0.417	23	0.412	24	0.352	35	0.428	21
3	150	0.211	61	0.401	26	0.390	28	0.369	32	0.320	41	0.406	25
4	200	0.167	70	0.379	30	0.363	33	0.336	38	0.287	47	0.374	31
5	250	0.105	81	0.347	36	0.336	38	0.309	43	0.249	54	0.347	36
6	300	0.053	90	0.320	41	0.314	42	0.293	46	0.222	59	0.325	40

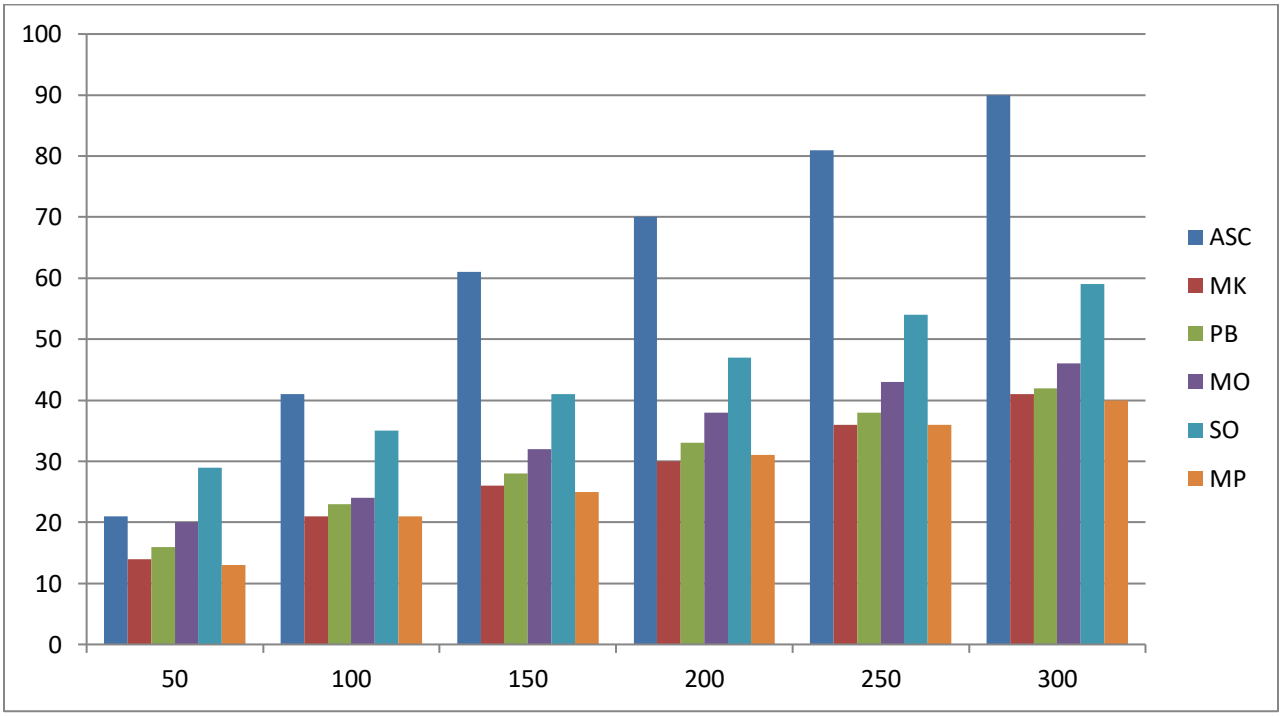

Figure-2 Hydrogen peroxide scavenging activity

In the graph AC-Ascorbic acid, MO- *Moringa olifera*, MK- *Murraya koenigii*, MP- *Mentha Piperita*, SO- *Spinacia oleracea*, PB- *Piper betel*

Catalase enzyme activity test

All the natural extracts at various concentrations 50, 100, 150, 200, 250, 300µg/ml are compared with the standard drug ascorbic acid. The control absorbance here is initial absorbance of hydrogen peroxide in the absence of any antioxidant drug. Decrease in absorbance after addition of drug indicates decrease in concentration of hydrogen peroxide which is an indication of reduction of hydrogen peroxide. The reduction of hydrogen peroxide is nothing but antioxidant activity. Decrease

in absorbance from the control is used for calculation of % Reduction of hydrogen peroxide. This indicates antioxidant effect of the drug.

Table-4 Data for catalase activity test **Control absorbance value-0.896**

Sno	conc	Ascorbic Acid		Murayya koenigii		Piper betel		Moringa oleifera		Spinacia oleracea		Mentha piperita	
		Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd	Abs	%Rd
1	50	0.6	33	0.769	14	0.759	15	0.712	21	0.651	27	0.779	13
2	100	0.547	39	0.713	20	0.691	23	0.668	25	0.589	34	0.715	20
3	150	0.494	45	0.682	24	0.653	27	0.621	31	0.543	39	0.685	24
4	200	0.43	52	0.625	30	0.604	33	0.563	37	0.495	45	0.621	31
5	250	0.384	57	0.576	36	0.565	37	0.491	40	0.439	51	0.579	35
6	300	0.332	63	0.542	40	0.543	39	0.496	45	0.405	55	0.539	38

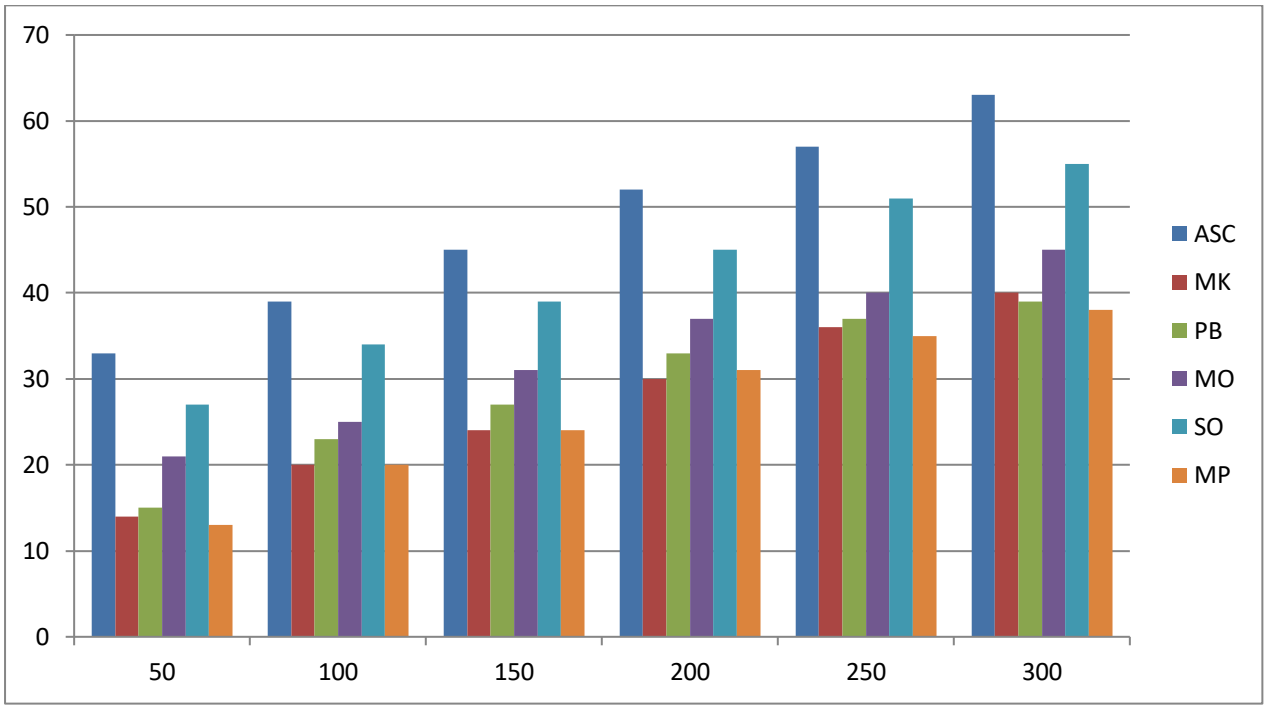

Figure-3 Catalase activity method

In the graph AC-Ascorbic acid, MO- *Moringa olifera*, MK- *Murraya koenigii*, MP- *Mentha piperita*, SO- *Spinacia oleracea*, PB- *Piper betel*

DISCUSSION

Reducing power assay

Reducing power is associated with antioxidant activity, compounds that reducing power indicates that they are electron donor and can reduce oxidized intermediates. So that, they can act as primary and secondary antioxidants. In this method, yellow colour of the test solution changes to various shades such as green and blue depending on the reducing power of the each compound.

The bar chart of the result shows that every drug possess antioxidant activity but at lesser potency than standard drug Ascorbic acid. Hydroalcoholic extract of Spinacia oleracia at 600µg dose produced approximately equal antioxidant effect to that of 500µg dose of standard.

ANOVA Of Reducing power assay						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Drugs	4814.667	5	962.9333	2.435477	0.057494	2.533555
Within Drugs	11861.33	30	395.3778			
Total	16676	35				

Results obtained from the reducing power assay are subjected to Single factor Anova. The results anova reveal that p value is slightly greater than 0.05 and f is value slightly lesser than critical value. This indicates the difference between the drugs in terms of reducing power assay is insignificant.

Hydrogen peroxide scavenging activity

Hydrogen peroxide itself is not highly reactive, but sometimes it can be toxic to cell because it gives rise to hydroxyl radical in the cells. Thus removing of hydrogen peroxide molecules is very important for antioxidant defense in cell or food systems.

The bar chart of the results obtained shows that every drug possess antioxidant activity but at lesser potency than standard drug Ascorbic acid. Hydroalcoholic extract of Spinacia oleracia at 300µg dose produced approximately equal antioxidant effect as that of 150µg dose of standard.

ANOVA of Hydrogen peroxide scavenging activity						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Drugs	5026.222	5	1005.244	5.057975	0.001768	2.533555
Within Drugs	5962.333	30	198.7444			
Total	10988.56	35				

Results obtained from the Hydrogen peroxide scavenging activity are subjected to statistical analysis using single factor Anova. The results of anova reveal that p value is lesser than 0.05 and f value is greater than the critical value. This indicates the difference between the drugs in terms of Hydrogen peroxide scavenging activity is significant and there is significant difference among the drugs.

Catalase enzyme activity

Enzymes are proteins that can act as biological catalysts. They help chemical reactions proceed more easily in the bodies of organisms. Hydrogen peroxide is produced naturally in organisms, but it is very harmful. Catalase and other enzymes helps to break down the hydrogen peroxide into water and oxygen.

The bar chart of the results obtained shows that every drug possess antioxidant activity but at lesser potency than standard drug Ascorbic acid. Hydroalcoholic extract of Spinacia oleracia at 300µg dose produced approximately equal antioxidant effect as that of 250µg dose of standard.

ANOVA of Catalase enzyme activity						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Drugs	2295.889	5	459.1778	4.637118	0.002972	2.533555
Within Drugs	2970.667	30	99.02222			
Total	5266.556	35				

Results obtained from the Catalase enzyme activity are subjected to statistical analysis using single factor Anova. The results of anova reveal that the p value is lesser than 0.05 and f value is greater than critical value. This indicates difference between the drugs in terms of Catalase enzyme activity is significant and there is significant difference among the drugs.

CONCLUSION

The hydro alcoholic extracts of *Mentha piperita***,** *Moringa oleifera, Spinacia oleracea, Piper betel, Murraya koenigii* have antioxidant activity. It is made clear from reducing power assay, hydrogen peroxide scavenging activity and catalase activity method which were performed using ascorbic acid as standard drug. Though the not equal or superior to standard drug used, they possess considerable antioxidant activity. All the drugs which were subjected to dtudy are natural food suppliments used in Indian kitchen. Regular use of these food suppliments in definite and desired quantities avoids oxidative stress. However their usefulness in pharmaceutical industry is to be investigated. Among all the five natural extracts *Spinacia oleracea* shows high antioxidant property when compared to *Mentha piperita, Murraya koenigii, Piper betel, Moringa oleifera.*

Spinacia oleracea > *Moringa oleifera* > *Murraya koenigii* > *Piper betel* > *Mentha piperita*.

REFERENCES

1. Abdul wadood, Mehreen Ghufran, Syed BabarJamal, Muhammad Naeem, Ajmal Khan , Rukhsana Ghaffar and Asnad. Phytochemical analysis of medicinal plants occurring in local area of mardan,et al. Biochem Anal Biochem.(2013) 2:144,doi;10.4172/2161-1009.1000144.

2. P. Jayanthi and P. Lalitha. Reducing power of the solvent extracts of Eichhornia crassipes (MART.) SOLMS . Int J pharm pharm sci,viol3, suppl 3,2011,126-128.

3. Sroka Z, Cisowski W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. Food Chem Toxicol.2003 Jun; 41(6):753-8.

4.Tadayuki Iwase, Akiko Tajima, and Yoshimitsu Mizunoe. A simple Assay for measuring catalase activity: A visual Approach Sci Rep.2013;3:3081.

5.Nurdin Rhaman,Nikmah Utami Dewi and Bohari. Phytochemucal and antioxidant activity of Avocado Leaf Extract(*Persea Americana* Mill.) Asian Journal of Scientific Research. (2018). Vol-11. Issue-3. Page no-357-363.

6. V. Lobo, A. Patil,[…], and N.Chandra.Free radicals,antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010 ;4(8):118-126.doi:10.4103/0973-7847.70902.

7. Jamuna,S.,S.Paulsamy and K. Karthika. Screening of invitro antioxidant activity of methanolic leaf and root extracts of Hypochaeris radicata L.(Asteraceae) Journal of applied pharmaceutical science 02(7);2012;149-154.

8.Paul Dowd and Zhizhen Barbara Zheng.On the mechanism of the anticlotting action of vitamin E quinine.Proc.Natl.Acad.92(1995) Vol. 92,pp.8171-8175.

9. Sunnyvale. Methods Optimization in Accelerated Solvent Extractionin Technical note. THERMO SCIENTIFIC (2013). 208: 1-4.

10. Azwanida NN. A REVIEW ON THE EXTRACTION METHODS USE IN MEDICINAL PLANTS,PRINCIPLE,STRENGTH AND LIMITATION. Med Aromat Plants. (2015).Vol-4. Issue-3. ISSN-2167-0412 MAP.

11. Manjulika yadav, sanjukta chatterjii, sharad kumar, gupta and geeta watal. PRELIMINARY PHYTOCHEMICAL SCREENING OF SIX MEDICINAL PLANTS USED IN TRADITIONAL MEDICINE. International Journal of Pharmacy and pharmaceutical Sciences. (2014)Vol 6. issue 5. ISSN-0975-1491.

12. THE INDIAN PHARMACOPOEIA COMMISSION, GHAZIABAD, Government of India, Ministry of health and family welfare. Indian Pharmacopoeia.(2010). Vol-1. Page no-560.

13. . Brown, W. E., Hu, J. C., & Athanasiou, K. A. (2016). *Ammonium–Chloride–Potassium Lysing Buffer Treatment of Fully Differentiated Cells Increases Cell Purity and Resulting Neotissue Functional Properties. Tissue Engineering Part C: Methods, 22(9), 895–903.*

14. Andrey V. Chernyshev, Peter A. Tarasov, Konstantin A. Semianov,Vyacheslav M. Nekrasov, Alfons G. Hoekstra, Valeri P. Maltsev.Erythrocyte lysis in isotonic solution of ammonium chloride:Theoretical modeling and experimental verification. Journal of theoretical bilology (2008) 251. 93-103.

15. Isabel C.F.R.Ferreira*, Paula Batista, Miguel Vilas-Boas, Lillian Barros. FREE-RADICAL SCAVENGING CAPACIY AND REDUCING POWER OF WILD EDIBLE MUSHROOMS FROM NORTHEAST PORTUGl:INDIVIDUAL CAP AND STIPE ACTIVITY. Food chemistry 100(2007)1511-1516.

16. Ahmed A. AI-Amiery, Yasameen K. AI-Majedy, Abdul Amir H. Kadhum, Abu Bakar Mohamad. Hydrogen Peroxide Scavenging Activity of Novel Coumarins SynthesizedUsing Different Approaches. PLOS|ONE (2015).

17. SAMUEL A. GOLDBLITH AND BERNARD E. PROCTOR. Photometric determination of catalase activity.J.Biol.chem.1950, 187:705-709.