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IN VITRO ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF CITRUS LIMON LINN. (LEAVES)

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ABSTRACT

Citrus limon linn belonging to *Rutaceae* family is highly reputed plant and has been widely employed in herbal medicine and aromatherapy but no work has been carried out on the antioxidant activity of the leaves extracts. The antioxidant property of methanolic extract of *Citrus limon* Linn. was evaluated by estimation of Total Flavonoid content (TFC), Free radical scavenging activity (DPPH- radical scavenging activity) and Total Phenolic content (TPC). The Total flavonoid content was measured by Aluminum chloride colorimetric method at 420 nm and was expressed as mg quercetein equivalent (QE)/gm dry plant material. The DPPH radical scavenging activity of *C.limon* Linn was estimated by Inhibitory concentration (IC_{50}) at 517 nm and the Total Phenolic content was analyzed by Folin - ciocalteu colorimetric method using gallic acid as standard (GAE), absorbance was reported at 765 nm. Results were reported as Total Flavonoid content was 3.73 mg/gm QE (quercetin equivalent), DPPH radical scavenging activity (IC_{50} value) was 310 µg/ml and Total Phenolic content was 1.96 mg/gm GAE (gallic acid equivalent). Thus from ongoing study it is concluded that leaves of *Citrus limon* Linn. possess significant Antioxidant activity and can be used for further research study.

KEYWORDS: Anti oxidant activity, *Citrus limon* Linn.

INTRODUCTION

Oxidative stress is an important risk factor in the pathogenesis of numerous chronic diseases. Free radicals and other reactive oxygen species are recognized as agents involved in the pathogenesis of sicknesses such as inflammation, diabetes, asthma, arthropathies, diabetes, Parkinson's and Alzheimer's diseases, atherosclerosis atherosclerosis as well as cancers. Reactive oxygen species are also said to be responsible for the human aging.

An antioxidant can be broadly defined as any substance that inhibits delays oxidative damage to a target molecule. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like Phenolics acids, Polyphenols and Flavanoids scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases. Herbal plants considered as good antioxidant since ancient times.

Citrus limon linn belonging to *Rutaceae* family is highly reputed plant and has been widely employed in herbal medicine and aromatherapy. *Citrus Limon* Linn. is well-known for its nutrition and health-promotion values. Traditional healers have used *citrus* species for centuries to treat various diseases. *Citrus* fruits are suggested to be a good source of dietary antioxidants. Different parts of *Citrus Limon Linn.* have been used for the treatment of various human ailments such as itches, cuts, ulcers, swellings, bilious fever, catarrh, eczema, antipsychotic etc. but no work has been carried out on the antioxidant activity of the leaves extracts.

Hence, the current study was designed to evaluate the antioxidant activity of methanolic extracts of *Citrus Limon* Linn. leaves. including by using DPPH scavenging assay, determination of total Phenolic content, determination of total Flavanoids contents and Ferric Reducing Power

MATERIALS AND METHODS

Plant material

The leaves of *Citrus limon* Linn. were procured and plants samples were identified and further confirmed by matching with the samples in the LWG herbarium of the National Botanical Research Institute, Lucknow, **Reference no. 97847**.

Preparation of Sample

Methanolic extract of leaf of *Citrus limon* Linn. was prepared through cold percolation by using 2 gm of powdered material in 100 ml of methanol. The filtered solution were pooled and dried for the evaporation of the solvent. Then they were dissolved in methanol at concentration and used for further analysis.

Total Antioxidant Activity and Free radical scavenging Assays

DPPH Radical Scavenging Assay

A solution of 0.135 mm DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1 ml of extract in methanol containing 0.02-0.1 mg of the extract. The reaction mixture was vortexes thoroughly and left in the dark at room temperature for 30 minutes the absorbance of mixture was measured spectrophotometrically at 517 nm ascorbic acid, Quercetin and Ascorbic acid were used as references. The ability to scavenge DPPH radicals was calculated by the following equation: of Liyana-Pathirana and Shahidi [Ez Ordon L. A. A. et al., 2006]. The result shown in **table 1and 2.**

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})] \times 100}{(\text{Abs control})}$$

Where: Abs control is the absorbance of DPPH radical + methanol;

Abs sample is the absorbance of DPPH radical + sample extract / reference.

Total Phenolic Content

Prepare a stock solution (1mg/ml) of extract in methanol. From the stock solution take suitable quantity of the extract into 25ml volumetric flask and add 10ml of water and 1.5ml of folin ciocalteu reagent keep the mixture for 5 min and then add 4ml of 20% Na_2CO_3 and make up to 25ml with distilled water. Keep the mixture for 30 min and record absorbance at 765nm. Total phenolic content was calculated as Gallic acid(mg/ml) using the following equation based on the calibration curve: y = 115.9x + 0.113, R^2 = 0.999, where y was the absorbance and x was the Gallic acid equivalent (mg/ml) [Bray et al., 1954]. The percentage of the total phenolic was calculated in triplicate with reference to the air dried drug. [N. Lakshmidevi et al., 2010]. The result shown in **table 3and 4**.

Total Flavonoid Content

Prepare a stock solution (1mg/ml) of extract in methanol. From the stock solution take suitable quantity of the extract into 25ml volumetric flask and add 10ml of water and 1.5ml of folin ciocalteu reagent keep the mixture for 5 min and then add 4ml of 20% Na_2CO_3 and make up to 25ml with distilled water. Keep the mixture for 30 min and record absorbance at 765nm. Total flavonoid content was calculated as Querecetin (mg/ml) using the following equation based on the calibration curve: y = 73.56x + 0.073, R^2 = 0.997, where y was the absorbance and x was the Querecetin equivalent (mg/ml) [Ez Ordon L. A. A. et al., 2006]. The percentage of the total phenolic was calculated in triplicate with reference to the air dried drug. [N. Lakshmidevi et al., 2010]. The result is shown in **5 and 6.**

Ferric Reducing Power Assay

The solution of the extracts (0.2–1.0 mg) in 1 ml of distilled water was mixed with 2.5 ml of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 ^{o} C for 20 min. Following this, 2.5 ml of 10% (w/v) Trichloroacetic acid was added and the mixture was then centrifuged at 1750 rpm for 10 min. A 2.5 ml aliquot of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) FeCl3; the absorbance of the mixture was read at 700 nm [Vani et al., 1997; Oyaizu M. 1986, Leelavinothan, 2007].The result Shown in table 7 and 8**.**

RESULT

DPPH Radical Scavenging Activity: Methanolic extract of *Citrus limon* leaves shows maximum inhibition of free radicals upto 62.63 % at a concentration of 500 µg/ml. while the two standards Ascorbic acid and Quercetin shows maximum inhibition of 71.91% & 79.69% at concentration of 5 ug/ml & 5 ug/ml. IC_{50} of sample was calculated as **385µg/ml**. while the two standards Ascorbid acid and Quercetin was calculated as **0.033 µg/ml** and **0.034 µg/ml.**

Table 1: Absorbance at various concentrations (mg/ml) of in-vitro antioxidant activity.

Concentration	0.02	0.04	0.06	0.08	0.1
Ascorbic acid	0.526	0.404	0.391	0.233	0.171
Quercetin	0.521	0.405	0.240	0.211	0.204
Leaf	0.449	0.354	0.308	0.236	0.201

Table 2: Percentage of Standards and Sample at various concentrations (mg/ml).

Concentration	0.02	0.04	0.06	0.08	0.1
Ascorbic acid	13.87%	33.74%	45.89%	61.81%	71.91%
Querecetin	1.54%	25.9%	41.81%	69.34%	79.69%
Leaf	5.54%	15.20%	32.75%	56.13%	62.63%

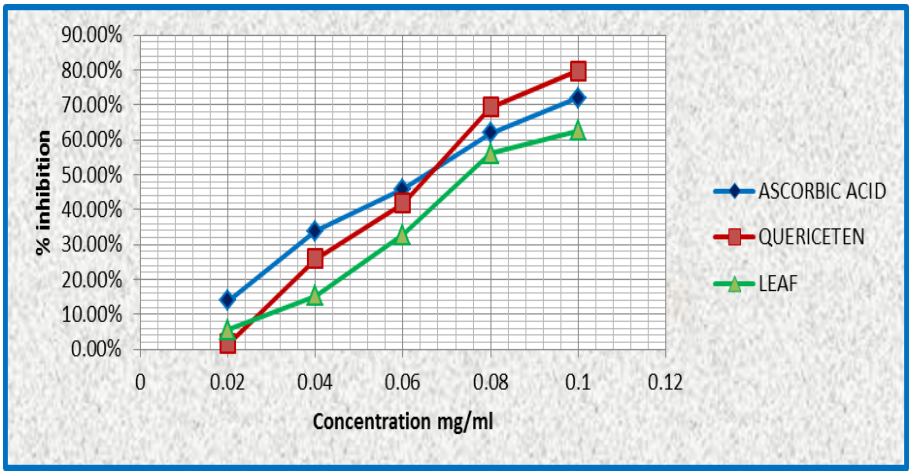

Figure 1: DPPH % free radical inhibition Vs. Concentration (mg/ml).

Total Phenolic Content

Table 3: Absorbance at various concentrations of standard and sample (mg/ml) of in-vitro antioxidant activity.

Concentration	0.0008	0.0016	0.0024	0.032	0.0040
Gallic acid	0.2108	0.2931	0.3893	0.4844	0.5790
Leaf *C. limon*	0.0008	0.0009	0.0009	0.0009	0.0009

Table 4: Preparation of calibration curve for phenolics content.

S. no.	Amount from stock (ml)	Dist. Water (ml)	Folin- ciocalteu's phenol reagent(ml)	20% sodium carbonate solution (ml)	Dist. Water (ml) Up to	Conc. (mg/ml)	Abso. At 765 nm
1	0.2	10	1.5	4	25	0.0008	0.2108
2	0.4	10	1.5	4	25	0.0016	0.2931
3	0.6	10	1.5	4	25	0.0024	0.3893
4	0.8	10	1.5	4	25	0.0032	0.4844
5	1	10	1.5	4	25	0.0040	0.579
6	Blank	10	1.5	4	25	-	-

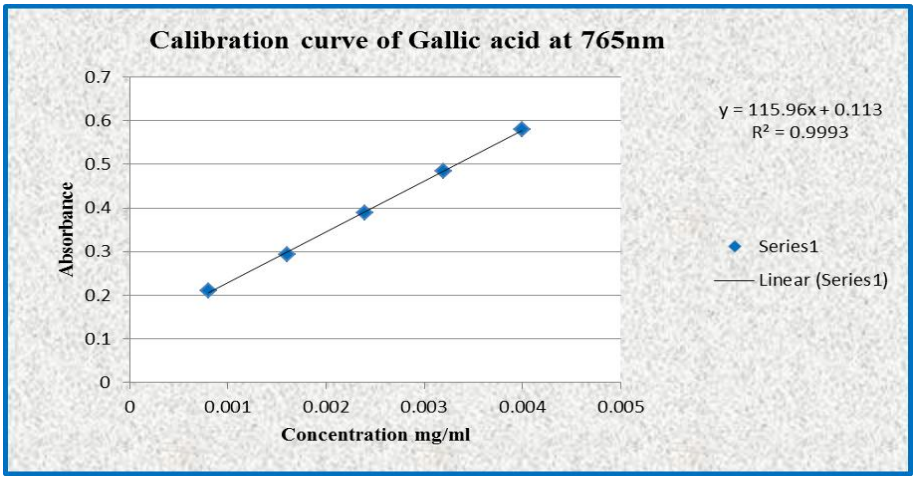

Figure 2: Calibration curve of Gallic acid showing phenolic content.

- Results obtained in the present study revealed that the level pf polyphenols in the methanolic extract of the leaves of *Citrus limon* Linn. was calculated as **4.81 ± 0.1068 mg/g GAE** (Gallic acid Equivalent).

Total Flavonoid Content

Table 5: Absorbance at various concentrations of standard and sample (mg/ml) of in-vitro antioxidant activity.

Concentration	0.004	0.008	0.012	0.016	0.020
Quericetin	0.3453	0.6692	0.9677	1.2711	1.5157
Leaf *C. limon*	0.0015	0.0018	0.0018	0.0018	0.0018

Table 6: Preparation of calibration curve for flavonoid content.

S. no.	Amount from stoke (ml)	2% aluminum chloride solution(ml)	Dist. Water (ml) up to	Conc. (mg/ml)	Abso. At 420 nm
1	0.2	0.5	10	0.004	0.3453
2	0.4	0.5	10	0.008	0.6692
3	0.6	0.5	10	0.012	0.9677
4	0.8	0.5	10	0.016	1.2711
5	1	0.5	10	0.020	1.5157
6	Blank	0.5	10	-	-

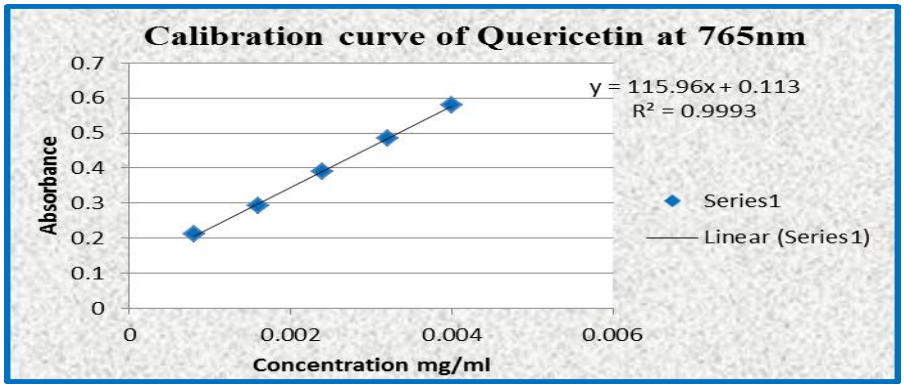

Figure 3: Calibration curve of Quericetin showing phenolic content.

- Results obtained in the present study revealed that the level flavonoids in the methanolic extract of the leaves of *Citrus limon* Linn. was calculated as**11.322 ± 0.0640 mg/g QE**(Quericetin Equivalent).

Ferric Reducing Power Assay

Table 7: Preparation of dilutions for calibration curve (std. is Ascorbic acid & Quercetin).

S. no	Volume Taken From Stock (ml)	Distill Water upto 1ml	Phosphate buffer (pH 6.6) + Potassium ferricyanide (ml)	Final Volume Taken (ml)	Distill water (ml)	Ferric chloride (0.1% w/v)	Absorbance at 700 nm	
							Ascorbic Acid	Quercetin
1	0.02	0.98	2.5 + 2.5	2.5	2.5	0.5	0.2363	0.2043
2	0.04	0.96	2.5 + 2.5	2.5	2.5	0.5	0.2488	0.2301
3	0.06	0.94	2.5 + 2.5	2.5	2.5	0.5	0.2899	0.2494
4	0.08	0.92	2.5 + 2.5	2.5	2.5	0.5	0.3222	0.2709
5	0.1	0.90	2.5 + 2.5	2.5	2.5	0.5	0.3539	0.2904
6	Control	1.0	2.5 + 2.5	2.5	2.5	0.5	0.223	0.180

Table 8: Absorbance at various concentrations (mg/ml) of in-vitro antioxidant activity.

Concentration	0.02	0.04	0.06	0.08	0.1
Ascorbic acid	0.2633	0.2488	0.2934	0.2899	0.3539
Quercetin	0.2043	0.2301	0.2494	0.2709	0.2904
Leaf	0.178	0.179	0.184	0.218	0.220

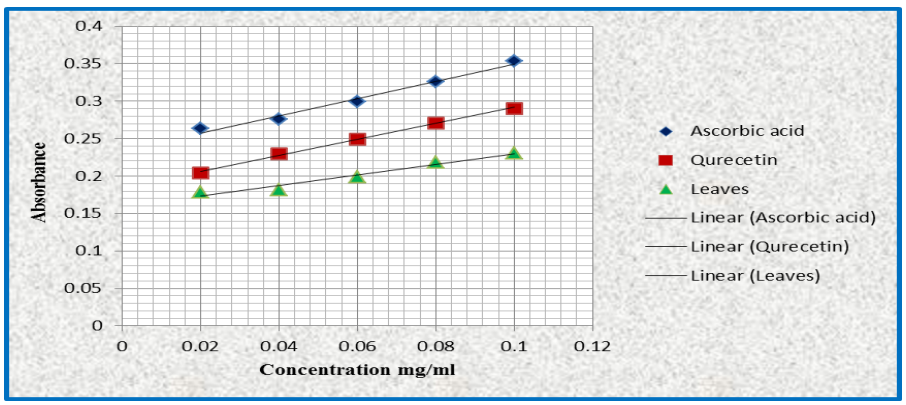

Figure 4: Reducing power of *Citrus limon* leaf extract at different concentrations (mg/ml).

- The reducing power of *Citrus limon* extracts using the potassium ferricyanide method is shown in **Figure 4.** The result indicates that the reducing ability of the extract increased with the concentration. as shown by the increasing optical density at **700 nm**.

DISCUSSION AND CONCLUTION

In-vitro DPPH free radical scavenging activity: In-vitro DPPH free radical scavenging activity of the methanolic extract of leaf of *Citrus limon* Linn. were compared with Ascorbic acid and Quericetin (standard used),. At a concentration of 0.1mg/ml the maximum scavenging activity of the leaf reached 62.63%. According to the results tabulated in **table1 and 2 Fig. 1.** Absorbance of control was 0.611.The stable radical DPPH has been used widely for the determination of primary anti-oxidant activity. The DPPH anti-oxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of anti-oxidants. The present study showed that IC_{50} value of sample is 385 µg/ml while that of Ascorbic acid and Quercetin is 0.033 µg/ml and 0.034 µg/ml respectively. Analysis by one way ANOVA shows that value of sample is comparable to that of Standards and hence it is stated that leaves of *Citrus limon* serve as good source of Anti oxidants.

Total Phenolic Content: In – vitro TPC activity of the methanolic extract of leaf of *Citrus limon* Linn. were compare with Gallic acid (standard used). Results obtained in the present study revealed that the level polyphenols in the methanolic extract of the leaves of *Citrus limon* Linn. was 4.81±0.1068 mg/g which was compared to Gallic acid. The result was tabulated in **table 3 and 4, Fig. 2.**

Total flavonoid Content: In – vitro TPC activity of the methanolic extract of leaf of *Citrus limon* Linn. were compare with Quericetin (standard used).Results obtained in the present study revealed that the level flavonoind content in the methanolic extract of the leaves of *Citrus limon* Linn. was 11.322±0.0640 mg/g which was compared to Quericetin. The result was tabulated in **table 5 and 6, Fig. 3.**

Medicinal plants are an important source of antioxidants. Natural anti-oxidants increase the anti-oxidant capacity of the plasma and reduce the risk of certain diseases. Polyphenols are the major plant compounds with anti-oxidant activity. Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids and flavonoids. It is reported that the phenolics are responsible for the variation in the anti-oxidant activity of the plant. They exhibit anti-oxidant activity by inactivating lipid free radicals or preventing decomposition of hydro peroxides into free radicals. Flavonoids are phenolic acids which serve as an important source of anti-oxidants found in different medicinal plants and related phytomedicies. The anti-oxidant activity of flavonoids is due to their ability to reduce free radical formation and to scavenge free radicals.

Ferric Reducing Power Assay: The reducing power of leaf of *Citrus limon* Linn.extracts using the potassium ferricyanide method is shown in **Fig. 4.** The result indicates that the reducing ability of the extracts increased with the concentration as shown by the increasing optical density at 700 nm. The result was tabulated in **table 7 and 8, Fig.4.**

Reducing power is associated with its anti-oxidant activity and may serve as a significant reflection of the anti-oxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary anti-oxidants.

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