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Assessment of Antioxidant Potential *of Holoptelia integrifolia* Roxb. Leaves by in *vitro*

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Abstract

Natural antioxidants present in parts of plants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Plant derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radicalscavengers, peroxide decomposers, enzyme inhibitors and synergists. *Holoptelia integrifolia* leaves contain Flavonoids, Triterpenoids like, Hexacosanol, Octacosanol, β-sitosterol& β-amyrin are present. Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert potent antioxidant actions. The Alcohol extracts of *H. integrifolia* leaves exhibited higher Antioxidant activity than Water extracts in DPPH, NO scavenging, H_2O_2 scavenging, ferric ion reduction and metal chelating *in vitro* antioxidant models. The present study revealed that assessment of the antioxidant potency by *in vitro* method of different extract of *Holoptelia integrifolia* which helps in the development of nutritional food supplement.

Keywords

Antioxidant, *Holoptelia integrifolia* Roxb, DPPH, Nutritional Food Supplement

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1. Introduction

Oxidation is defined as removal of electrons from a molecule. Oxidation can damage tissue by modifying a number of molecular species, such as lipids, proteins and nucleic acid. Oxidation is one of the destructive processes, wherein it breaks down and damages various molecules (Mason, 1987). Antioxidant plays an important role in removing such free radicals. Free radicals reaction is an important pathway in a wide range of unrelated biological systems. Amongst the many ways to chemically injured and kill cells, an important class of reaction is that producing free radicals intermediates which trigger a network of multifarious disturbances because of ubiquity of molecular oxygen in aerobic organism and its ability to accept electron. Oxygen derived free radicals are often mediators or products of normal pathological or toxic free radical reaction (Mohan, 2005). Natural antioxidants present in parts of plants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Plant derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radicalscavengers, peroxide decomposers, enzyme inhibitors and synergists. Antioxidant action focuses on phenolic compounds such as flavonoids. Fruits and vegetables contain different antioxidant compounds, such as vitamin C, vitamin E and carotenoids, whose activities have been established in recent years. Flavonoids, tannins and other phenolic constituents, Present in food of plant origin are also potential antioxidant (Alessio, 1997).*Holoptelia integrifolia is* useful in vital conditions of kapha and pitta, inflammations, dyspepsia, flatulence, colic, helminthiasis, vomiting, skin diseases, leprosy, diabetes, hemorrhoids and rheumatism (Orient Longman, 2005).*Holoptelia integrifolia* leaves contain Flavonoids, Triterpenoids like, Hexacosanol, Octacosanol, β-sitosterol & β-amyrin are present. Stem bark

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contains Alkaloids like, Friedelin, Friedelin-3-β-ol and Holpotelin A and B. Heart wood contains β-sitosterol, hedergenin (Chopra, 1957). Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert potent antioxidant actions (Sivakumar, 2012).The present study was carried out to assess the antioxidant potency of different extract of *Holoptelia integrifolia* which helps in the development of nutritional food supplement.

2. Material and Methods

Collection and authentication of plant: Fresh leaves of *Holoptelia integrifolia* Roxb was collected from college campus of The M. L. Gandhi Higher Education Society, Modasa. Identification of plant was carried out by Dr. M. S. Jangid, Botanist, Sir P. T. Science College, Modasa. Voucher specimens [PHCOG/1224/2008/01] were deposited in Pharmacognosy museum, department of Pharmacognosy, Shri B. M. Shah College of Pharmaceutical Education and Research, Modasa.

Preparation of Extracts: Shade-dried and powdered leaves of *Holoptelia integrifolia* was extracted with (95%) ethanol and to get the alcoholic extract. The leaf powder was macerated with 400 ml distilled water and adds few drops of chloroform for preventing microbial growth. Seven days with occasional shaking to get the aqueous extract. All the extracts were concentrated under reduced pressure using rotary evaporator and the residue was dried in desiccators over anhydrous calcium chloride.

Qualitative chemical tests: All the extracts were tested to know the different constituents present in them by the standard procedures. The extracts were tested for sterols (Finar, 1975), alkaloids, (Cromwell, 1955), triterpenes, saponins, tannins (Kokate, 1996), flavonoids (Geinssman, 1955), carbohydrates (Hawks, 1971).

3. *In-Vitro* Antioxidant Activity

a) DPPH scavenging activity

Take the different concentration of alcoholic and aqueous extracts of *Holoptelia integrifolia*. Butylated Hydroxy Toluene (BHT) and Ascorbic acid were used as Standard. Extracts and standard in 1ml of ethanol and add 4 ml of 100 µM DPPH in methanol. The mixture was allowed to stand at room temperature for 20 minutes and absorbance was measured at 517 nm by colorimeter. The absorbance of control was first noted at 517nm excluding ascorbic acid or extracts. Scavenging activity was expressed as the inhibition percentage calculated using the formula(Singh, 2002). Each experiment was carried out in triplicate and results were expressed as mean % antiradical activity ± SD.

$$\text{Percentage Anti radical activity} = [(A_c-A_s)/A_c] \times 100$$

Where; A_c is the absorbance of the control and A_s is the absorbance of extract/standard

b) Nitric oxide scavenging activity

Add 0.5 ml SNP (10mM) in Phosphate Buffered Saline (pH 7.4) was mixed with different concentrations of extract of alcoholic and aqueous extracts of *Holoptelia integrifolia* in ethanol, Ascorbic acid as standard and incubated at 25^0C for 180 min, then Griess reagent (1% sulfanilamide, 0.1% naphylethylenediamine dihydrochloride and 3% Phosphoric acid) volume equal to incubated solution was added. The absorbance was immediately measured at 546 nm using colorimeter. Absorbance of control excluding ascorbic acid or extracts. The Nitric oxide scavenging activity was calculated from the formula (Sreejayan, 1996)

$$\text{Percentage Nitric oxide scavenging activity} = [(A_c-A_s)/A_c] \times 100$$

Where, A_c is the absorbance of the control and A_s is the absorbance of extract/standard

c) Scavenging of Hydrogen peroxide

Take different concentrations of extract of alcoholic and aqueous extracts of *Holoptelia integrifolia* and standard ascorbic acid solution in methanol (1 ml). Add 2 ml hydrogen peroxide containing phosphate buffer solution. Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used. The percentage inhibition activity was calculated from (Kumaran, 2007)

$$\text{Percentage inhibition activity} = [(A_c-A_s)/A_c] \times 100$$

Where; Acis the absorbance of the control and A_s is the absorbance of extract/standard

d) Reduction of ferric ions

Take 1ml ortho-Phenanthroline (0.005g in 10 ml methanol), 2 ml ferric chloride 200 µM (3.24 mg in 100 ml distilled water) & 2 ml of different concentrations of alcoholic and aqueous extracts of *Holoptelia integrifolia* extract and standard ascorbic acid solution 100, 200, 300, 600, 800, 1000 µg/ml in 2ml of methanol of the extract was incubated at 50^oC temperature for 10 min. Measured the absorbance at the 510 nm. Take the absorbance of control excluding ascorbic acid or extracts. The experiment was performed in triplicate. (Srinivasan, 2007)

Percentage Ferric ion reducing activity = $[(A_c-A_s)/A_c] \times 100$

Where; A_c is the absorbance of the control and A_s is the absorbance of extract/standard

e) Metal chelating Assay

The chelating activity of the extracts of ferrous ion Fe^{2+} was measured. Take different concentration of 0.5 ml alcoholic and aqueous extracts of *Holoptelia integrifolia* or EDTA, 1.6 ml distilled water and 0.05 ml $FeCl_2$ (2 mM). After 30 s, add 0.1 ml ferrozine (5mM). After 10 min at room temperature, take absorbance at 562 nm. Take the absorbance of control excluding EDTA or extracts. The chelating activity of the extracts for Fe^{2+} was calculated as percentage metal chelating (Zhonggao, 2005).

Percentage Metal chelated= $[(A_c-A_s)/A_c] \times 100$

Where; A_c is the absorbance of the control and A_s is the absorbance of extract/standard

f) Total antioxidant capacity

100 µg of alcoholic and aqueous extracts of *Holoptelia integrifolia* or ascorbic acid (as standards) were taken in 0.1 mL of alcohol, mixed in an eppendroff tube with 1.9 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. Samples were cooled to room temperature; the absorbance was measured at 695 nm against a blank. A typical blank solution contained 1.9 ml of reagent solution and the appropriate volume of the same solvent used for the sample and was incubated under the same conditions as the rest of the samples. Total antioxidant capacity was calculated using standard graph of Ascorbic acid (100-1000 µg/ml) and express equivalents to Ascorbic acid in µg per mg of extract. The experiment was conducted in triplicate (Mruthunjaya K, 2008)

g) Statistical analysis

Data are reported as mean of three determinations. The results obtained were statistically analyzed by annova Student's t-test using a significance level of $P < 0.05$.

4. Results and Discussion

a) *DPPH free radical scavenging activity. In-Vitro* Antioxidant was carried out on Alcohol and Water extracts of *H. integrifolia* using Ascorbic acid and BHT as standard antioxidant and results are shown in figure 1

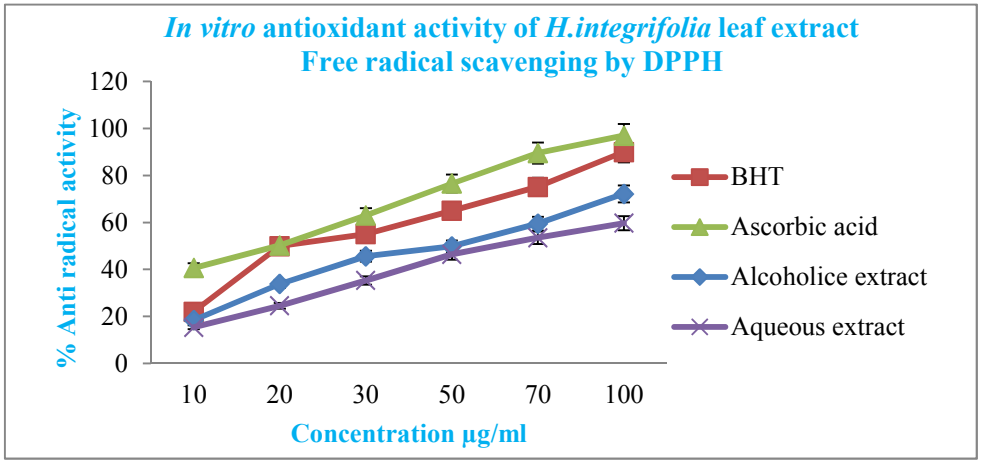

Fig. 1. Antioxidant activity *H. integrifolia* extracts in DPPH model

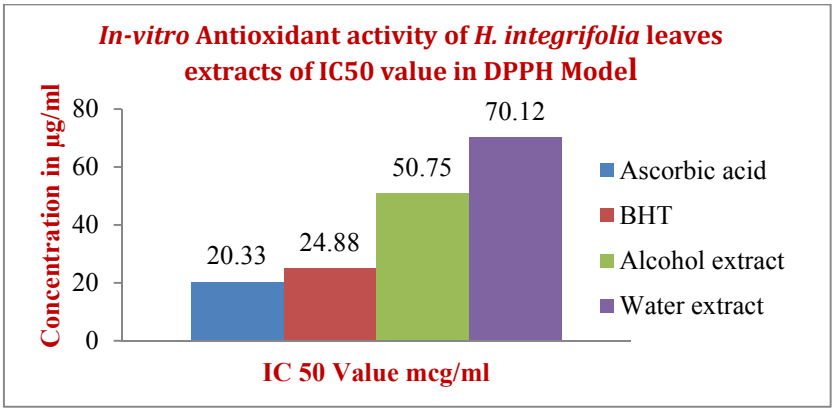

Fig. 2. IC50 Value of *H. integrifolia* leaves extracts in DPPH model

The Alcohol and Water extracts of *H. integrifolia* had shown dose dependant antioxidant activity in DPPH model.

Figure 1illustrates a significant ($p < 0.05$) decrease in the concentration of DPPH radicals due to the scavenging ability of *Holoptelia* extract. The IC_{50} values for Alcohol, Water extract, BHT and Ascorbic acid are shown in figure 2

IC_{50} values for Alcohol, Water extract of *H. integrifolia*, BHT and Ascorbic acid had shown 50.75±1.42, 70.12±0.55, and 24.88±2.14 and 20.33±1.98 µg/ml respectively. Flavonoids, tannins and glycosides, the common constituents present in the extracts of *H. integrifolia* may attribute for antioxidant activity.

b) *Nitric oxide scavenging activity: In-Vitro* Antioxidant was carried out on Alcohol and Water extracts of *H. integrifolia* using Ascorbic acid as standard antioxidant and results are shown in figure no. 3.It reveals that a significant ($p < 0.05$) dose response relationship is found in the nitric oxide free radical scavenging activity in *Holoptelia* extract.

The Alcohol and Water extracts of *H. integrifolia* had shown dose dependant antioxidant activity in NO model. The IC_{50}values for Alcohol, Water extract, and Ascorbic acid are shown in figure 4

The IC_{50} value ascorbic acid, Alcohol and Water extracts of *H. integrifolia* was 200.76±0.13, 272.85±0.033 and 574.80±0.043µg/ml respectively. Flavonoids, tannins and glycosides the common constituents present in both extracts of *H. Integrifolia* may be responsible for antioxidant activity.

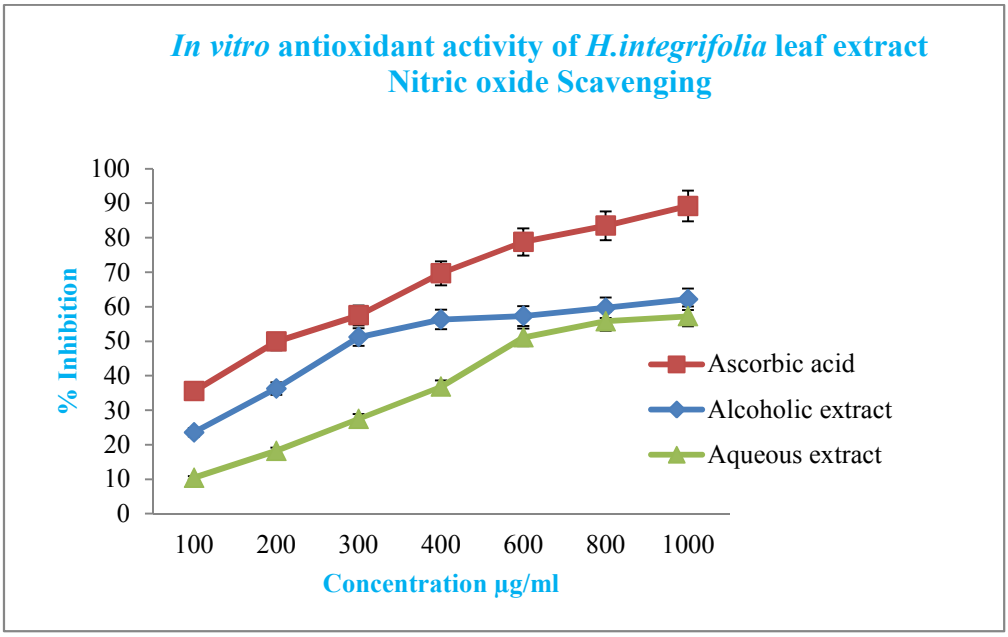

Fig. 3. Antioxidant activity *H. integrifolia* extracts in NO model

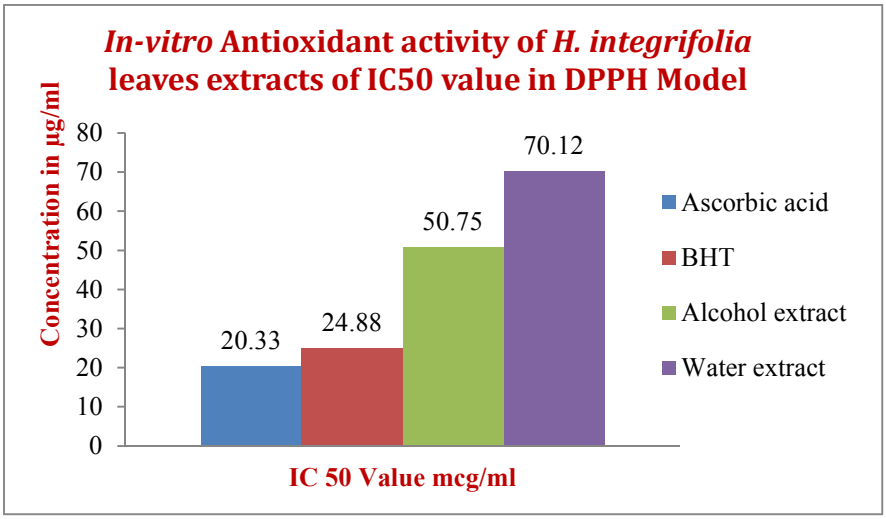

Fig. 4. IC50 Value of *H. integrifolia* leaves extracts in NO model

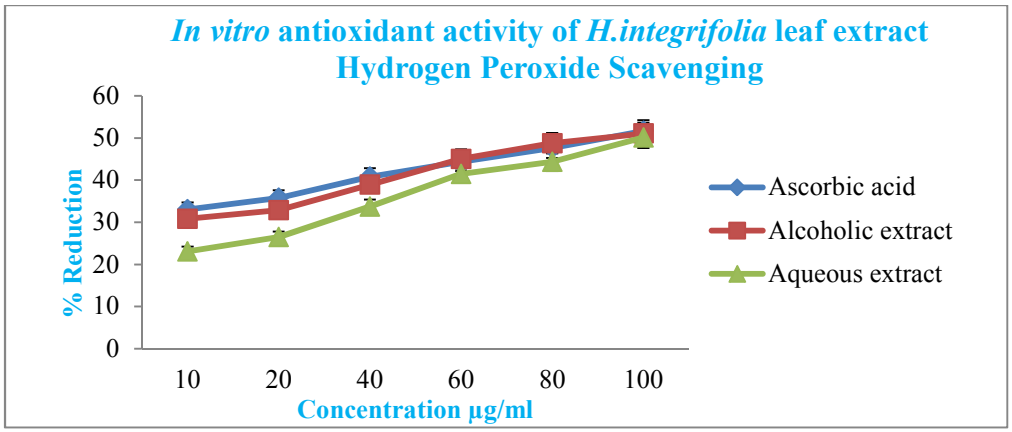

Fig. 5. Antioxidant activity *H. integrifolia* extracts in H_2O_2 model

c) *Hydrogen Peroxide scavenging activity:* Percentage reduction of hydrogen peroxides was carried out on Alcohol and Water extracts of *H. integrifolia* and ascorbic acid as standard and results are shown in Figure 5. It reveals that a significant ($p < 0.05$) dose dependent response was found in the hydrogen peroxide scavenging activity in *Holoptelia* extract.

The Alcohol and Water extracts of *H. integrifolia* had shown dose dependant antioxidant activity in H_2O_2 model. The IC_{50} values for Alcohol, Water extract, and Ascorbic acid are shown in figure no. 6

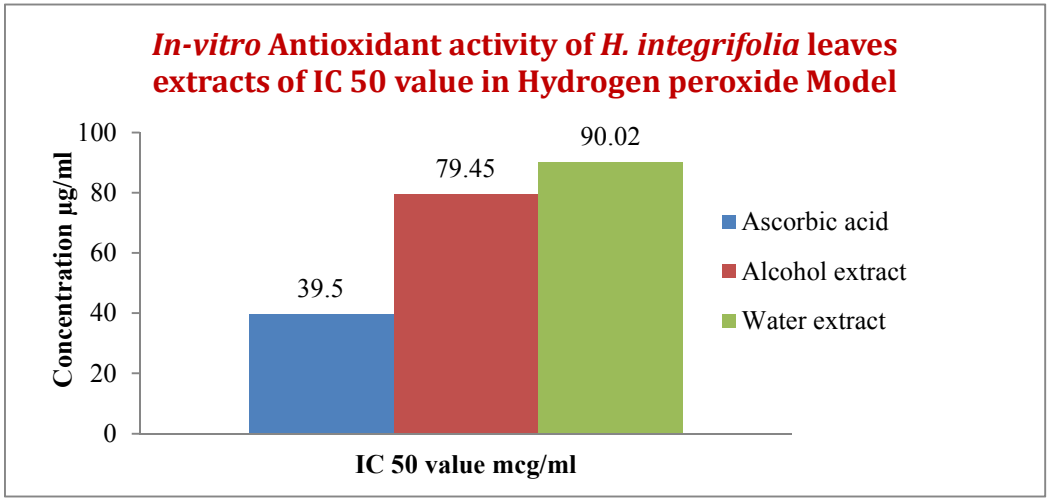

Fig. 6. IC_{50} Value of *H. integrifolia* leaves extracts in H_2O_2 model

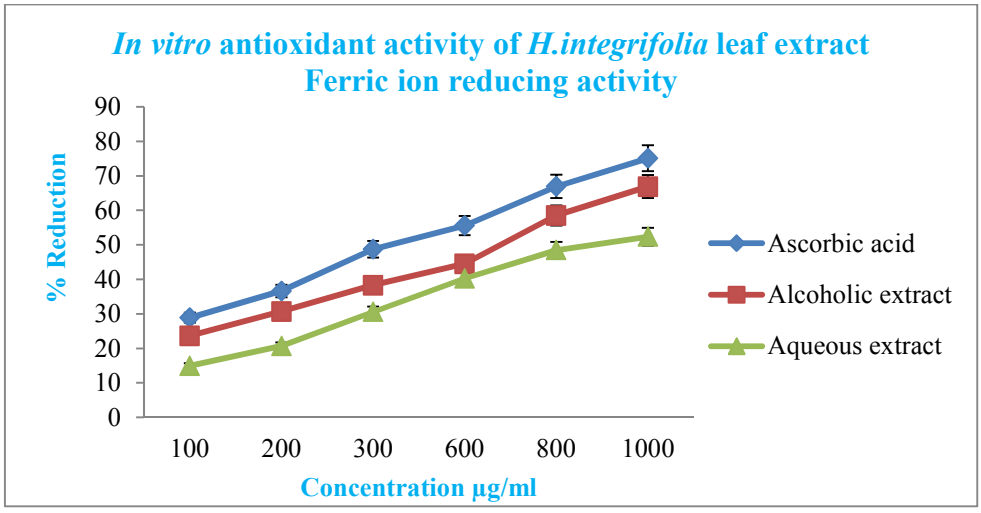

Fig. 7. Antioxidant activity *H. integrifolia* extracts in ferric ion model

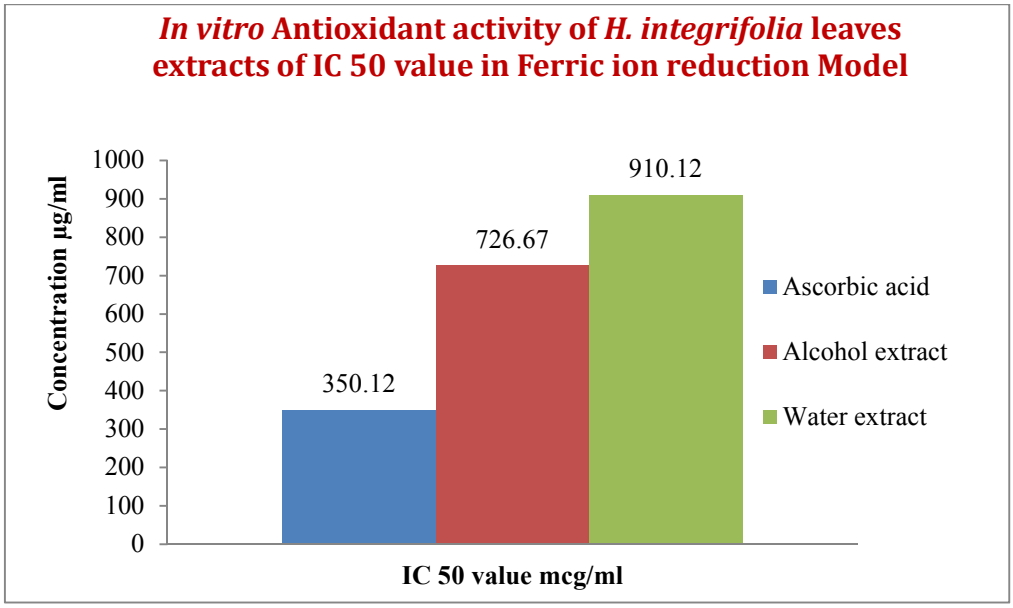

Fig. 8. IC_{50} Value of *H. integrifolia* leaves extracts in ferric ion model

IC_{50} values 39.50±0.05, 79.45±0.19 and 90.02±0.50µg/ml for ascorbic acid, Alcohol and Water extract of *H. Integrifolia* leaves respectively.

d) *Reduction of ferric ions:* Percentage reduction of ferric ions was carried out on Alcohol, Water extracts of *H. integrifolia* and ascorbic acid as standard and results are shown in Figure 7It reveals that reducing power of *H. Integrifolia* extract was statistically significant ($p < 0.05$).

The Alcohol and Water extracts of *H. integrifolia* had shown dose dependant antioxidant activity in ferric ion reduction model. The IC_{50} values for Alcohol, Water extract, and Ascorbic acid are shown in figure no. 8

The IC_{50} value determined for ascorbic acid, Alcohol and Water extracts of *H. integrifolia* was350.12±0.05, 726.67±0.15 and 910.12±0.23 µg respectively.

e) *Metal chelating Assay:* Percentage metal chelating activity was carried out on Alcohol, Water extracts of *H. integrifolia* and EDTA as standard antioxidant and results are shown in figure no.9.It reveals that metal chelating power of *H. Integrifolia* extract was statistically significant ($p < 0.05$).

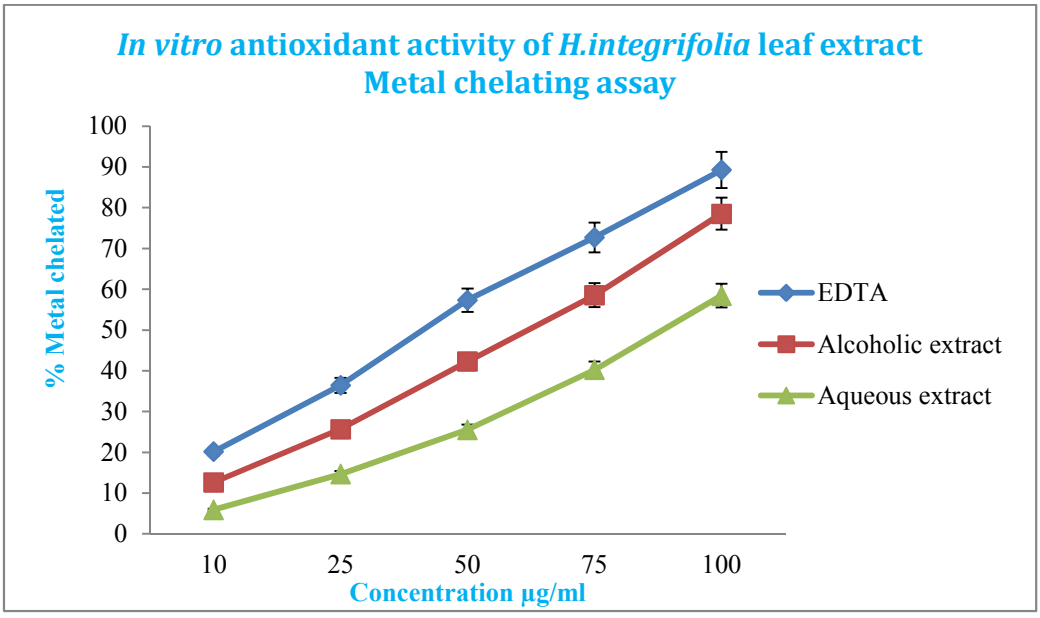

Fig. 9. Antioxidant activity *H. integrifolia* extracts in metal chelating model

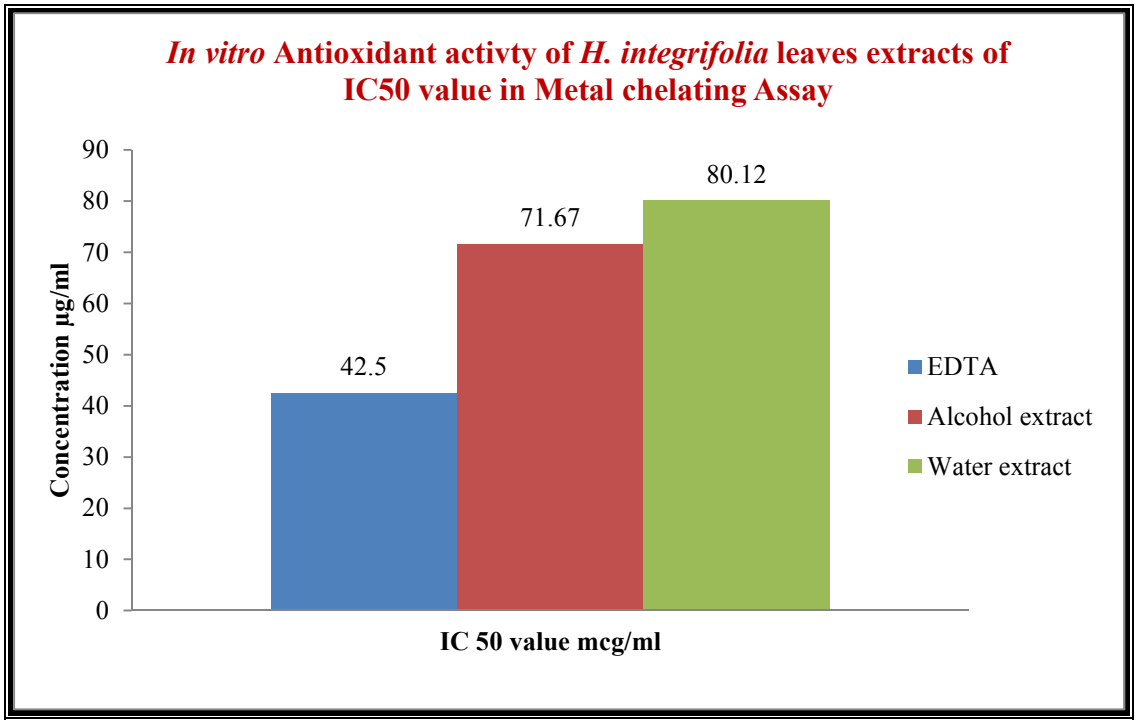

Fig. 10. IC50 Value of *H. integrifolia* leaves extracts in metal chelating model

The Alcohol and Water extracts of *H. integrifolia* had shown dose dependant antioxidant activity in metal chelating model. The IC_{50} values for Alcohol, Water extract, and EDTA are shown in figure no.10

The IC 50 value of EDTA, Alcohol and Water extract of *H. Integrifolia*leaves were found to be 42.50±0.15, 71.67±0.09 and 80.12±0.08 µg respectively. The Alcohol extract of *H. Integrifolia*leaves exhibited higher metal chelating capability than Water extract.

f) *Total antioxidant capacity:* The total antioxidant capacity was carried out on Alcohol, Waterextracts of *H. integrifolia* leaves using BHT and ascorbic acid as standard antioxidant and results are shown in table no. 1

Total antioxidant capacity of Alcohol, Water extracts of *H. integrifolia*, ascorbic acid and BHT were found to be 290.50±0.62, 310.10.±0.89, 197.22±0.81 and 225.00+1.05 µg equivalent to ascorbic acid/mg respectively, indicating higher antioxidant capacity in Alcohol extracts of *H. Integrifolia* leaves than Water extract.

Table No. 1. Total antioxidant capacity of *H. Integrifolia*leaves

Extract/Standard	Total antioxidant capacity in µg
Ascorbic acid	297.22±0.81
BHT	225.00±1.05
AlcoholExt.	190.50±0.62
Water Ext.	110.10±0.89

g) IC_{50} value of *H. integrifolia* leaves extracts in antioxidant model

Above data are summarized using IC_{50} values of Alcohol and Water extracts of *H. integrifolia* leaves in different antioxidant model in table no.2&fig. 11

Table No. 2. IC_{50} value of *H. integrifolia* leaves extracts in antioxidant model

Antioxidant Model	Std. Antioxidant	Standard	Alcohol	Water
		IC_{50} Value µg/ml		
DPPH	BHT Ascorbic acid	24.88±2.14 20.33±1.98	50.75±1.42	70.12±0.55
Nitric oxide	Ascorbic acid	200.76±0.13	272.85±0.33	574.80±0.43
Hydrogen Peroxide	Ascorbic acid	39.50±0.55	79.45±0.19	90.02±0.50
Ferric Ions Reduction	Ascorbic acid	350.12±0.35	726.67±0.15	910.12±0.23
Metal chelating	EDTA	42.50±0.15	71.67±0.91	80.12±0.08

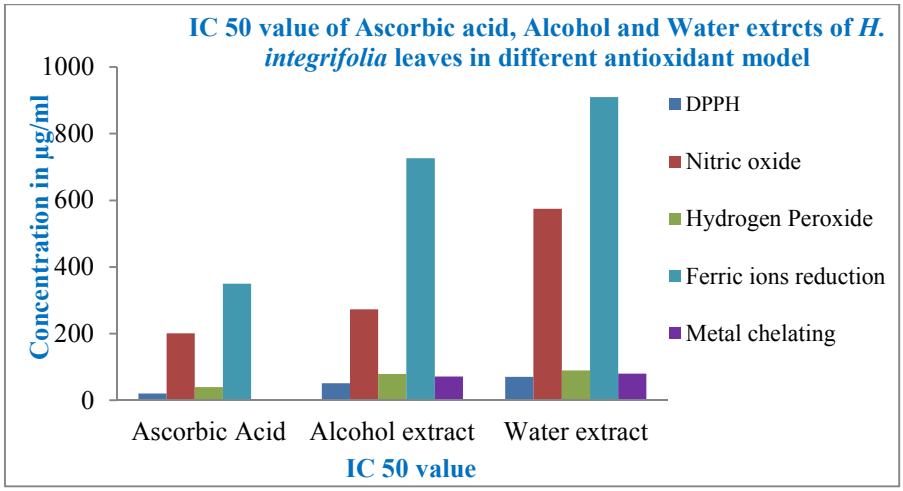

Fig. 11. IC $_{50}$ values of Ascorbic acid, Alcohol & Water extracts of *H. integrifolia* leaves.

IC $_{50}$ values of ascorbic acid in DPPH, nitric oxide, hydrogen peroxides and ferric reduction model were 20.33±1.98, 200.76±0.13, 39.50±0.05 and 350.12±0.05 respectively. IC $_{50}$ values of Alcohol extracts of *H.integrifolia* leaves were exhibited in DPPH model 50.75±1.42µg, nitric oxide model 272.85±0.033 µg, hydrogen peroxide model 79.45±0.19 µg, ferric ions reduction model 726.67±0.15 µg and metal chelating model 71.67±0.09 µg, indicating Alcohol extracts of *H. integrifolia* leaves had higher antioxidant activity in DPPH model. IC $_{50}$ values of Water extracts of *H. integrifolia* leaves were exhibited in DPPH model 70.12±0.55µg, nitric oxide model 574.80±0.043µg, hydrogen peroxide model 90.02±0.50µg, ferric ions reduction model 910.12±0.23µg and metal chelating model 80.12±0.08 µg, indicating Water extracts of *H. integrifolia* leaves had higher antioxidant activity in DPPH model. The Alcohol extracts of *H. integrifolia* exhibited higher Antioxidant activity in all performed *in vitro* antioxidant models than Water extracts. The IC $_{50}$ values for ascorbic acid, Alcohol and Water extracts of *H. integrifolia* leaves vary in different antioxidant models suggesting antioxidant activity depends on selected antioxidant model.

5. Conclusion

The Alcohol extracts of *H. integrifolia* leaves exhibited higher Antioxidant activity than Water extracts in DPPH, NO scavenging, H_2O_2 scavenging, ferric ion reduction and metal chelating *in vitro* antioxidant models. In the present study, the nutritional composition of *H. integrifolia* leaves revealed that rich source of polyphenols, carbohydrate, flavonoids, tannins and energy. In addition, the leaves extracts proved to be higher in polyphenol compounds and an antioxidant property. The present study revealed that assessment of the antioxidant potency by *in vitro* method of different extract of *Holoptelia integrifolia* which helps in the development of nutritional food supplement.

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