Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 230-235
Received: 19-10-2017
Accepted: 22-11-2017

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Antioxidant activity of some extracts of *Iris ensata*

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Abstract
Introduction: *Iris ensata* is a rare medicinal plant found in Kashmir valley.
Methods: Antimicrobial activity of the of the plant exracts were carried out against against various bacterial and fungal strains like: *E. coli (MTCC 407*), *P. aeruginosa (MTCC 139), S. aureus (MTCC 96), B. subtilis (MTCC 441*), *K. pneumonia (MTCC 49*); *A. niger (MTCC1344*), *P. crysogenum (MTCC 947), C. albicans (MTCC), T. rubrum (MTCC 8469), E. floccosum (MTCC 613), M. canis* (MTCC 296). Various antioxidant methods were used for evaluation of free radical scavenging activity of the plant extracts, IC_{50} values were found to be lowering with increased polarity.
Results: The plant under study was found to have broad spectrum antimicrobial activity against various bacterial and fungal strains The plant also shows a significant radical scavenging activity with IC50 values of 98.94 124.63, 98.63, 191.88, 100.79 µg/ml in methanolic extract.
Conclusion: The plant can also serve as potential alternative to treat various diseases, as the plant was found to have broad spectrum antimicrobial potential.

Keywords: DPPH; *P.kashmirians*; Phytochemicals

1. Introduction
In their definition of the term, Halliwell & Gutteridge state that an antioxidant is 'any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate [37]. This definition includes compounds of a non-enzymatic as well as an enzymatic nature. Natural antioxidants can be classified as primary (chain-breaking) antioxidants, which can react directly with radicals and convert them into stable products, or as secondary (preventive) antioxidants, which can lower the rate of oxidation by different mechanisms [38]. Primary antioxidants most often act by donating a hydrogen atom, while secondary antioxidants may act by binding metal ions able to catalyze oxidative processes, by scavenging oxygen, by absorbing UV radiation, by inhibiting enzymes or by decomposing hydroperoxides [39]. Both of these classes of antioxidants function synergistically to neutralize effects of ROS [40].
Free radicals generation is a normal phenomenon in biological system. Sometimes due to over generation, body's defence mechanism is not able to remove them, and as a result a condition called oxidative stress is developed in the body. The free radicals are the chemical species, which have an unpaired electron and are thus vary unstable and reactive. In order to attain stability they react with their neighbouring atoms to gain the electrons resulting the generation of new free radicals,which in turn attack to other nearby molecules causing a web of reactions. Aerobic organism use of oxygen to oxidize food and obtain the energy,a phenomenon essential for their sustenance. But during this oxidation process the oxygen molecule itself get reduced and from an intermediate called as the reactive oxygen species (ROS). The role of free radical reaction in the biology has become an area of intense interest. It is generally accepted that free radicals play an important role in the development of tissue damage and pathological events in living organisms. There is increasing interest in the natural antioxidants contained in the medicinal and the dietary plants, which are the candidates for the prevention of oxidative stress or damage.
Iris ensata belongs to Family *Iridaceae* that comprises of about eighty genera and about 1500 species.

2. Material and Methods
Iris ensata was collected from its natural source in Kashmir. After identification of the plant from dept. of botany Islamia college, Srinagar, the leaves of the plant were taken and thoroughly washed with tap water, rinsed a few times in distilled water and then dried in shade.The dried leaves were taken and cut out into small pieces, powdered in a grinder and then extracted with petroleum ether, ethyl acetate and methanol.

2.1 Preparation of Plant Extracts

A standardized solvent extraction protocol was used. 50 g of plant powder was fed to a Soxhlet extractor fitted with a 0.5 L round-bottom flask and a condenser. Extraction was carried out serially with different solvents using petroleum ether, ethyl acetate and methanol. The extraction was executed on a water bath for 12 hrs with 0.4 L of each solvent. After completion of extraction, the solvent was distilled off in a rotary evaporator at 35-45 ºC. The dried extracts were weighed to determine the yield of soluble constituents. All the extracts of the plant were stored under refrigerator (4 ºC), until used for further analyses.

2.2 Phytochemical screening

The phytochemical screening tests of the different plant extracts were performed by using standard procedures (Sofowora 1993; Ayoola *et al*. 2008).

2.3 Determination of Total of phenolic content

The total content of soluble phenolic compounds in plant extracts was determined with Folin Ciocalteau reagent (FCR) according to the method described in (Chang *et al.* 2002; Roy et al, 2010). Gallic acid was used as a standard. 0.5 ml of each extract with a concentration of 5 mg /ml was separately mixed with Folin–Ciocalteu reagent (0.2 N, 2.5 ml) and aqueous Na_2CO_3 (1 M, 2 ml) solution. The reaction mixture was allowed to stand at room temperature for 15 min. The absorbance was measured at 765 nm using a UV–visible spectrophotometer. The calibration curve (Slope = 57.44 ± 1.10; R2 = 0.997) was prepared using solutions of gallic acid (standard) in methanol: water mixture (50:50, v/v) with concentrations ranging from 0–30 µg ml-1. The total polyphenol content was expressed in terms of milligram of gallic acid equivalent per gram of dry mass (mg GAE g-1). Three replicates were performed for each sample concentra concentration to check the reproducibility of the experimental result and to get more accurate results.

2.4 Determination of total flavonoid content

The Aluminium chloride colorimetric method was used for flavonoid content determination of each extract (Roy et al, 2010; Mcdonald et al 2001). 0.5 ml of each extract (5 mg ml-1) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The reaction mixture was kept at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a UV–visible spectrophotometer. The calibration curve (Slope = 65.68 ± 0.76; R2 = 0.999) was recorded by using quercetin (standard) solutions in methanol with concentrations ranging from 0 to 20 µgml-1. The total flavonoid content was expressed in terms of milligram of quercetin equivalent per gram of dry mass (mg QE g^{-1})

2.5 Evaluation of antioxidant activity

The antioxidant activities were performed using assays viz. DPPH radical scavenging assay, Ferric ion reducing assay, FRAP assay, Hydrogen peroxide scavenging assay and Lipid per-oxidation assay. The methods of these assays are described below:

2.5.1 Assay for *in vitro* DPPH- free radical scavenging activity (DPPH assay)

DPPH free radicle scavenging activity is one of the basic reproducible method for antioxidant screening of numerous compounds. Free radicle scavenging potentalities were tested against the methonolic solution of DPPH. DPPH accepts an electron or hydrogen radicle to become a stable, diamegnetic molecule. It can be oxidised only with difficulty and reversibily. Because of its odd electron, 1,1- diphenyl-2-picryl hydrazyl shows a strong absorption band at 517nm its solution appearing in a deep violet colour. As the electrons becomes paired off, the absorption vanishes and the resulting decolourization is stiochimetric with respect to the electrons taken up. Antioxidant reacts with DPPH and converts into 1,1- diphenyl-2- picryl hydrazine. The purple colour of DPPH changes to yellow (decolorization), indicating the scavenging efficacy of added substance. The change in absorbance at 517nm has been used to measure antioxidant property. The assay was standardized using ascorbic acid as standard. The DPPH assay was performed as described by (Bozin *et al.*, 2001). The reaction mixture 4ml contained, 0.1ml of (50, 100, 150, 200, 400µg/ml) various concentration of lectin samples in 0.1M Phosphate buffer (pH 7.2), 3.9 ml of DPPH (.025gm/l). solution was addedto all above test tubes. Incubated at room temperature in the dark for 30 minutes. A blank determination with 0.1 ml of methanol solution instead of standard treated similary was maintained. For control 0.1 ml of methanol with 3.9 ml of DPPH solution was used. The optical density was measured at 517 nm using a spectrophotometer.

Scavenging activity of DPPH free radicle in percent was calculated according to equation.

DPPH radical scavenging activity (%) = 1-A/B×100

Where: A= Absorbance of blank (control)

B= Absorbance of test sample.

IC_{50} values were calculated from the plot of inhibition percentage against concentration.

2.5.3 Assay for *in vitro* ferric reducing antioxidant power (FRAP assay)

For FRAP assay, the modified Ruch *et al*. (1989) method was adopted. It is based on the principle of reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ complex at low pH which gives blue color. Plant extracts of (0.1 ml) at various concentrations (50, 100, 150, 200, 400 µg/ml) were allowed to react with 2.9 ml of FRAP solution for 30 min in the dark condition. Readings of the colored product (Ferrous tripyridyltriazine complex) were taken at 593 nm. The blank was prepared by using distilled water in place of sample/standard.

2.5.4 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out (Feerreira et al, 2010). A solution of H_2O_2 was prepared in phosphate buffer (0.1 M, pH 7.4). The extract at different concentrations in 3.4 ml phosphate buffer was added to 0.6 ml of H_2O_2 solution (0.6 ml, 43 mM). The absorbance value of the reaction mixture was recorded at 230 nm.

% H_2O_2 radical scavenging activity = 1 – Atest/ Acontrol x 100

Where: A**control** is the absorbance of the control. A**test** is the absorbance in the presence of the sample.

The yields of various extracts of the plants under study are given in Table 1. All the extracts were obtained as dark-green semi-solid material.

Table 1: Yields of various extracts of plant material (in grams).

Name of Plant	Pet ether Extract	Ethyl acetate Extract	Methanol Extract
Iris ensata	3.44	6.3	8.6

3.1 Phytochemical screening

Different methods were followed to determine qualitatively the presence of phytochemical constituents present in the plant methanol extract. The amount of crude extracts varied among the solvents used. Under the present study, the methanol extract (8.6 g) showed higher yield. The qualitative phytochemical screening of crude extracts of *Iris ensata* revealed that alkaloids, phenols, anthraquinones, and flavonoids were present. Saponins, glycosides, and +tannins were absent in both the RP and AP extracts while terpenoids were present in only the RP extract (Table 2).

Table 2: Phytochemical analysis of different secondary metabolites present in the aerial extracts of *Iris ensata*

Phytochemical constituents Extract	Pt. ether	Ethyl acetate	Methanol.
Alkaloids	+	+	+
Glycosides	+	+	+
Anthraquinones	+	+	+
Saponins	–	+	+
Tannins	–	–	-
Terpenoids	–	+	+
Flavonoids	+	+	+
Phenolic compounds	+	+	+

(+) = present, (−) = absent

Total phenolic and total flavinoidal contents in the extracts were expressed as GAE and Quercetin equivalents are presented in Table 3 and 4. Methanol extract of *I. ensata* registered highest value of phenolic and flavinoidal contents among its extracts. However, Pet-ether extracts of the plant species under study presented low phenolic and flavinoidal contents as expected.

Table 3: Total Phenolic content of the plant under study, expressed as gallic acid equivalents in µg/ml for 100 g of extract.

Total Phenolic Content			
Plant name	**Gallic Acid Equivalent) (µg/ml for 100 g of extract)**		
Extract	Pet. ether Extract	Ethylacetate Extract	Methanol
Iris ensata	23.83	665.08	844.56

Table 4: Total flavonoid content of the plants under study, expressed as Quercetin equivalents in µg/ml for 100 g of extract.

Total FlavonoidContent			
Plant name	**Quercetin Equivalent) (µg/ml for 100 g of extract)**		
Extract	Pet. ether Extract	Ethylacetate Extract	Methanol
Iris ensata	8.87	664.24	1004.84

3.2 Antioxidant Activity

3.2.1 DPPH radical scavenging assay

DPPH assay is one of the most widely used method for screening antioxidant potential. DPPH being a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecule. The reduction capability of DPPH radical was determined by decrease in its absorbance at 517 nm, which is induced by different antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants because of the reaction between antioxidant molecule and radical progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. The activities obtained as a function of various concentrations of different extracts are presented (Figure 1). It is clear from the figure that the methanolic extract shows higher DPPH radical scavenging activity. The IC_{50} values of the extracts for this activity were determined from the graph and values are given in the Table 5 for all extracts.

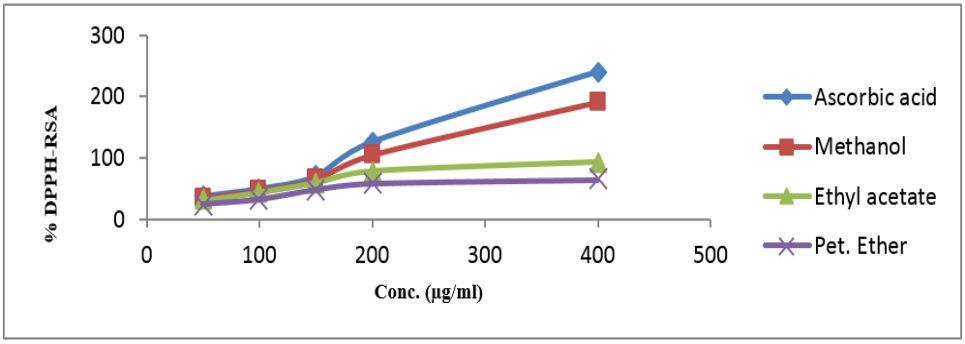

Fig 1: % DPPH Radical Scavenging Activity of various extracts of under study plants and standard (Ascorbic acid).

3.2.2 Ferric (Fe^{3+}) reducing power assay

The reducing ability to convert Fe^{3+} to Fe^{2+} is also an indirect evidence for the antioxidant activity of an extract or a compound (Matsushige *et al*., 1996). In the ferric reducing antioxidant power assay, the antioxidants i.e. the reducing species present in the extract causes the reduction of the Fe^{3+} ferricyanide complex to form Fe^{2+} ions; this reaction was monitored spectrophotometrically by recording the absorbance of the reaction mixture at 700 nm (Prasad *et al*., 2010). The reducing power characteristics of different extracts is shown in (Figure 2). From the figure it is clear that, out of all three extracts of the plants studied, methanolic extract has highest ferric ion reducing activity at different concentration, followed by ethyl acetate extract and least for pet. ether extract. The reducing power increases with increasing the concentration of extracts in the solutions. IC_{50} value is shown in Table 5.

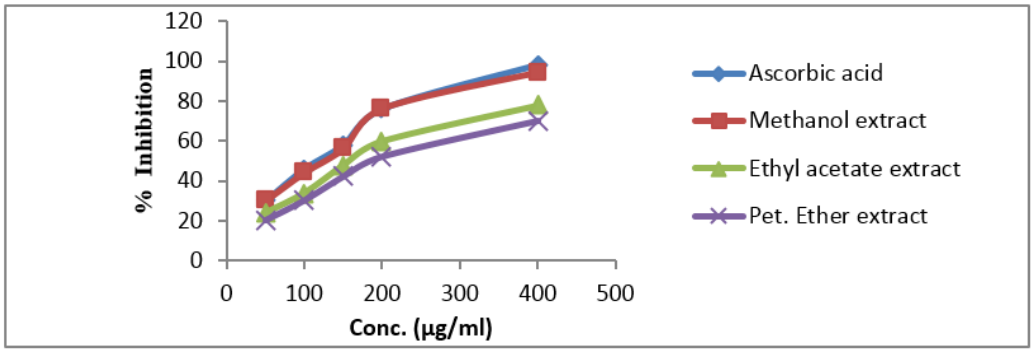

Fig 2: Ferric reducing activity of different extracts of under study plant and standard Ascorbic acid

3.2.3 FRAP

The ferric reducing antioxidant power (FRAP) assay measures the reducing ability of antioxidants against the oxidative effects of reactive oxygen species. Electron donating antioxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine (TPTZ), whereby an intense blue Fe^{2+}–TPTZ complex with an absorbance maximum at 593 nm is formed. Increasing absorbance indicates an increase in reductive ability. Among the different samples isolated from the studied plant at various concentrations (50, 100, 150, 200, 400 µg/ml) were examined. From Figure 3 it is clear that there was a concentration dependent increase in reducing activity in all extracts and methanol extracts possesses highest activity among all the extracts. IC_{50} value is presented in Table 5.

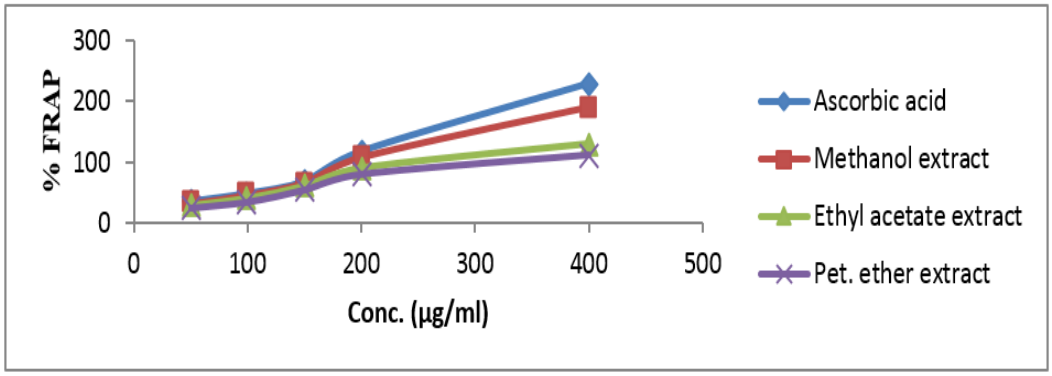

Fig 3: FRAP activity of different extracts of under study plant and standard Ascorbic acid

3.2.4 Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agents and can inactivate a few enzymes directly by the oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membrane rapidly, once inside the cell, H_2O_2 can probably react with every possible molecule in living organism especially with DNA, Proteins and Lipids and this may be the origin of many of toxic effects [27]. Hydrogen peroxide scavenging activity percentage of various extracs is presented in graph (Figure 4) and IC_{50} value is presented in (Table 5). The extracts showed the concentration dependent scavenging as compared with standard ascorbic acid.

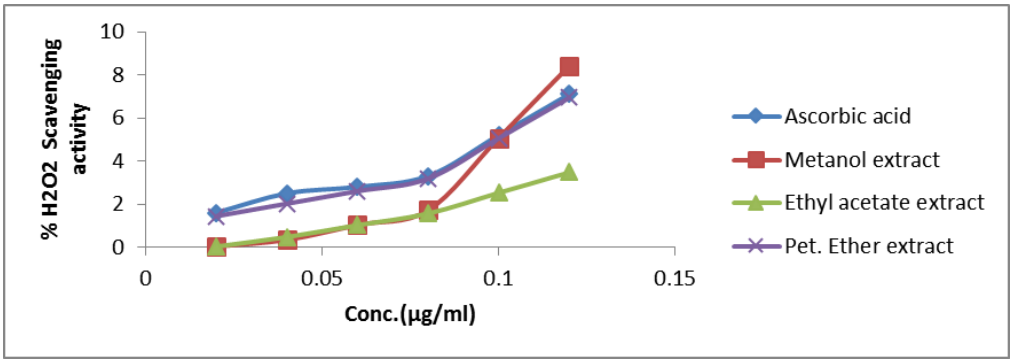

Fig 4: Hydrogen peroxide scavenging activity of various plant extracts and standard Ascorbic acid.

3.2.5 Lipid per-oxidation Method

Lipids Peroxidation has been a major problem for the shelf stability of foods. Due to oxidation of lipids, lipid hydroperoxides formed in the food systems are cleaved or polymerized to form various secondary products. These products are responsible for the inferiority of the food quality such as deterioration of taste and flavor (Spanier *et al*., 1992; Jensen *et al*., 2001) and decreased nutritional value (Ames, 1983). Lipids Peroxidation activity percentage of various extracs is presented in graph (Figure 5) and IC_{50} value is presented in (Table 5). The extracts showed the concentration dependent activity as compared with standard ascorbic acid.

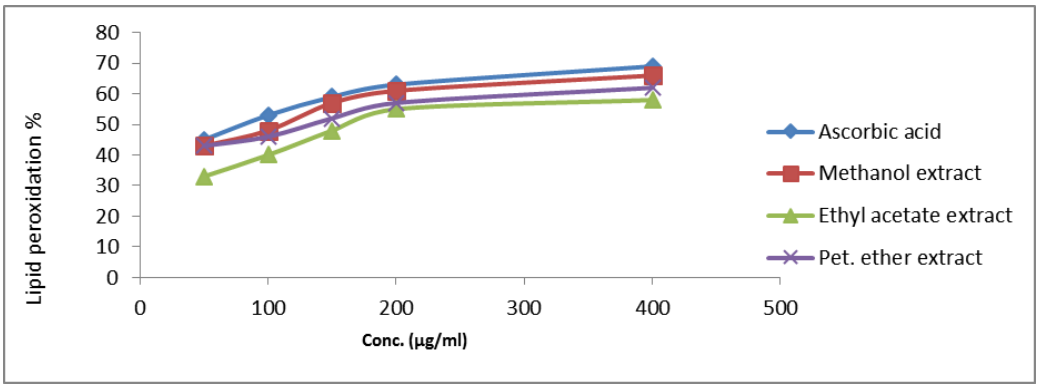

Fig 5: Lipid peroxidation activity of various plant extracts and standard Ascorbic acid.

Table 5: IC50 values of various extracts under different assays of plant under study.

Plant extract	IC50 (µg/ml of extract)				
	DPPH	Ferric reducing assay	FRAP	H_2O_2	Lipid peroxidation assay
Petroleum ether extract	223.69	226.66	138.17	330.98	229.55
Ethyl acetate extract	114.65	188.94	118.49	279.56	143.33
Methanol extract	98.94	124.63	98.63	191.88	100.79
Ascorbic acid	89.73	112.94	77.20	165.88	56.45

4. Conclusion

Iris ensata is a rare medicinal plant of Kashmir, before this work no such study has been conducted on this plant. The plant shows remarkable antioxidant potential and can be used for prevention or treatment of various oxidative related diseases. The plant can also serve as potential alternative to treat various diseases, as the plant was found to have broad spectrum antimicrobial potential.

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