ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 8, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

# EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY AND ESTIMATION OF TOTAL PHENOL AND FLAVONOID CONTENT OF ETHANOLIC LEAF EXTRACT OF *IRIS KASHMIRIANA*

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***Received: 04 March 2017, Revised and Accepted: 15 May 2017***

**ABSTRACT**

**Objective:** The main aim of this study was to determine the *in vitro* antioxidant activity of *Iris kashmiriana* ethanolic leaf extract and also total phenol and flavonoid content was evaluated.

**Methods:** Total phenol content (TPC) was determined by Folin–Ciocalteu method, total flavonoid content (TFC) was estimated by aluminum trichloride spectrophotometer method. Furthermore, antioxidant activity was revealed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, hydrogen peroxide ($H_2O_2$) scavenging activity, and reducing power assay.

**Results:** The ethanolic leaf extract of *I. kashmiriana* showed TPC of 13.25±0.57 µg/100 µg gallic acid equivalents and TFC of 33.61±3.37 µg/100 µg rutin equivalents. The DPPH assay revealed $IC_{50}$ of 0.418 mg/ml and for $H_2O_2$ radical scavenging $IC_{50}$ was 0.476 mg/ml for the plant extract while as reducing power assay revealed concentration-dependent absorption values which clearly determine the antioxidant property of plant.

**Conclusion:** From the results, it is apparent that *I. kashmiriana* ethanolic leaf extract possessed potential antioxidant activity which can be used to cure wide range of diseases.

**Keywords:** *Iris kashmiriana*, Total phenol content, Total flavonoid content, 1,1-diphenyl-2-picrylhydrazyl, Hydrogen peroxide.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i8.18268

## INTRODUCTION

Plants are the natural source of medicine and have been used since time to cure various ailments or diseases. The medicinal property of the plants is due to the presence of various bioactive constituents such as alkaloids, phenols, flavonoids, terpenoids, saponins, triterpenes, and tannins. The biological activities of such compounds are being investigated through different screening techniques [1]. It is commonly accepted that medicines derived from plant products are safer than their synthetic counterparts [2]. The ingestion of vegetables, fruit and herbs is coupled with the prevention of several bactericidal, antiviral, anti-inflammatory, analgesic and anticarcinogenic disorders, and due to their antioxidant activities [3,4]. The plant products such as fruits, leaves, seeds, and oils possess antioxidant property as they contain flavonoids, phenolics, tannins, coumarins, lignans, curcumanoids, xanthons, and terpenoids [5]. Phenols and flavonoids have been shown to acquire an essential antioxidant activity toward free radicals [6]. Rancidity of lipid and lipid containing is reduced with the use of antioxidants. They reduce the formation of toxic oxidation, maintain nutritional quality and enhance the life of food products [7].

Therefore, there is a growing demand day by day of the substances exhibiting antioxidant properties and which are part of human and animal food or used as specific preventative pharmaceutical product [8]. Hence, keeping in view the importance of antioxidants, this study, we planned to determine the *in vitro* antioxidant activity of ethanolic leaf extract of *Iris kashmiriana*.

## METHODS

### Sample collection and authentication

*I. kashmiriana* shoot was collected in the month of August (Village Lar, District Ganderbal, Jammu and Kashmir, India). Dr. Akhtar H. Malik (Curator, Centre for Biodiversity and Taxonomy, University of Kashmir) identified and authenticated the plant. A voucher specimen, bearing No. 2079 KASH was submitted to the same department for future reference.

### Preparation of sample

The leaves were detached from the plant, washed firstly with tap water to remove foreign matter and finally were washed with distilled water twice. Leaves were shade dried for about 2 weeks and then powdered with the help of electric grinder (Philips). The powder was packed in the polythene bags for further use, and the percentage loss was determined.

### Extraction procedure

In the Soxhlation process, 20 g of leaf powder was extracted using 90% ethanol as a solvent for 24 hrs with the help of Soxhlet Assembly (J-Sil, 50/42, Borosil glass). The extract was filtered and subjected to vacuum rotary evaporator (Scientech) to obtain a semi-solid extract. The extract was subjected to organoleptic evaluation, and the percentage yield of the extract was calculated, stored at −4°C.

### Phytochemical analysis

The crude leaf extract of *I. kashmiriana* was subjected to preliminary phytochemical analysis for presence or absence of bioactive constituents, according to standard procedures [9,10].

### Determination of total phenol content (TPC)

Folin–Ciocalteu method [11] was used to determine the TPC. Calibration was done with a standard gallic acid calibration curve. Different concentrations gallic acid (10-100 µg/ml) was prepared in methanol. Test sample (100 µg/ml) was prepared in methanol. A volume of 0.5 ml test sample was added to 2 ml Folin–Ciocalteu

reagent (1:10 in Deionized Water). Neutralization of mixture was brought by adding 4 ml of sodium carbonate solution (7.5% w/v). The final solution was incubated at room temperature for 30 minutes with intermittent shaking to develop color. At 765 nm, absorbance of colored solution was taken using double beam spectrophotometer (ultraviolet-visible analyst - 0001) using methanol as blank. Standard curve gallic acid was prepared, and line of regression was obtained (Fig. 1). TPC of the herb was calculated and expressed as µg/mg gallic acid equivalent.

**Determination of total flavonoid content (TFC)**

Aluminum trichloride spectrophotometer method [12] was used to determine TFC of plant, using rutin as standard. Various concentrations of rutin (10-100 µg/ml) were prepared in methanol. Furthermore, test sample (100 µg/ml) was prepared in methanol. A volume of 0.5 ml of test sample was diluted with 2 ml distilled water. 0.15 ml of $NaNO_2$ solution (5%) was then added and after 6 minutes, 0.15 ml of $AlCl_3$ (10%) was added, allowed to stand for 6 minutes. To the mixture, 2 ml of NaOH solution (1 mM) was added. Finally, the volume of reaction mixture was made 5 ml by diluting it distilled water and allowed to stand for 15 minutes. Taking methanol as blank, at 510 nm, absorbance of reaction mixture was obtained. TPC was determined from a calibration curve and expressed as µg/mg rutin equivalent (Fig. 2).

***In vitro* antioxidant activity**

*1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay [13]*

Various concentrations of the extract and the ascorbic acid were made in methanol. To 2 ml of extract or standard, add 1 ml of methanolic 0.1 mM DPPH and the reaction mixture was incubated at 37°C for 20 minutes. Absorbance was measured at 517 nm against blank using double beam spectrophotometer and % inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{Ac - As}{Ac} \times 100$$

Ac and As denotes absorbance of control and test sample, respectively.

*Hydrogen peroxide ($H_2O_2$) scavenging assay*

$H_2O_2$ scavenging activity of *I. kashmiriana* leaf extract was determined according to standard procedure [14] with slight modifications. Different concentration of the test sample was made in distilled water. 40 mM of $H_2O_2$ solution was made in phosphate buffer (pH 7.4). 2 ml of test sample and 0.6 ml of $H_2O_2$ were mixed well and incubated at room temperature for 10 minutes and then absorbance was measured against the blank (phosphate buffer without $H_2O_2$). Ascorbic acid was used as a standard. Percentage of $H_2O_2$ scavenging was calculated as:

$$\% \text{ Inhibition} = \frac{Ac - As}{Ac} \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of plant sample. $IC_{50}$ was determined from the regression curve of % inhibition.

*Reducing power assay*

Ferric reducing power assay [15] is used to determine the antioxidant property of plant. Diverse concentrations of test sample were made. 0.5 ml phosphate buffer (0.2 M, pH 6.6) was added to 0.5 ml of different concentrations of test sample, followed by simultaneous addition of 0.5 ml of potassium ferricyanide (0.5 ml, 1% w/v). At 50°C, reaction mixture was incubated for 20 minutes. Then, 1.5 ml of trichloroacetic acid solution (10% w/v) was added to reaction mixture after cooling, to terminate the reaction. Followed by addition of 0.5 ml ferric chloride (0.1% w/v) and absorbance was taken at 700 nm. Absorbance of reaction mixture is directly proportion to the reducing power, which is directly related to the antioxidant property.

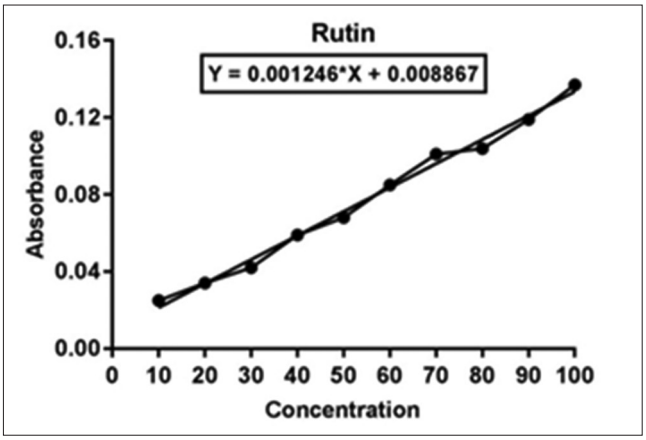


**Fig. 1: Gallic acid standard curve**

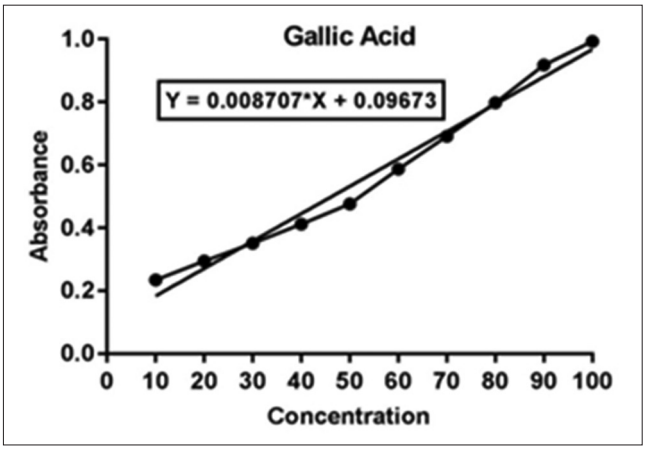


**Fig. 2: Rutin standard curve**

**RESULTS**

As, *I. kashmiriana* leaves were shade dried, the calculated percentage loss was 83.8%. Further, on complete Soxhlation of plant sample, an extractive value of 2.9 g was determined with percentage yield of 14.5%. The extract so obtained was dark green in color, bitter in taste, odor like lemon and was sticky in touch. The preliminary phytochemical analysis of crude extract of *I. kashmiriana* revealed the presence of almost all phytoconstituents except alkaloids as expressed in Table 1. The quantitative estimation of phenolics and flavonoids revealed ethanolic leaf extract of *I. kashmiriana* contained 13.25±0.57 µg/100 µg gallic acid equivalent of phenolics while as TFC was 33.61±3.37 µg/100 µg rutin equivalent as indicated in Fig. 3.

*In vitro* antioxidant activity results showed diverse concentrations of plant sample scavenges DPPH in a dose-dependent manner (Fig. 4). Results were expressed as % inhibition and were standardized with the ascorbic acid curve (Fig. 5). The $IC_{50}$ of *I. kashmiriana* leaf extract was 0.418 mg/ml while that of ascorbic acid 0.399 mg/ml. However, for $H_2O_2$ radical scavenging, $IC_{50}$ was 0.476 mg/ml and percentage inhibition showed gradual increase, i.e., concentration dependent scavenging activity by the plant sample (Fig. 6). Furthermore, reducing power assay showed steady increase in the absorption values (Table 2), indicating potential antioxidant property of plant extract. The 100 µg/ml concentration showed minimum absorption value of 0.192 nm while as 500 µg/ml concentration corresponded to maximum value of absorption 0.755 nm.

**DISCUSSION**

The secondary metabolites of the plant such as phenols, flavonoids, alkaloids, and terpenes are known to be biologically active constituents,

**Table 1: Phytochemical analysis of *I. kashmiriana* leaf extract**

| Phytochemicals | Phytochemical tests | Crude leaf extract of *I. kashmiriana* |
|---|---|---|
| Alkaloids | Mayer's test | - |
| | Wagner's test | - |
| | Hager's test | - |
| | Dragendroff's test | - |
| Terpenoids | Salkowski test | ++ |
| | Liebermann Burchard's test | - |
| Flavonoids | Lead acetate test | ++ |
| | Alkaline reagent test | ++ |
| | Shinoda test | ++ |
| Carbohydrates | Molisch's test | ++ |
| | Fehling's test | - |
| | Benedict's test | ++ |
| | Barfoed's test | - |
| Glycosides | Keller Kiliani test | ++ |
| | Borntrager's test | ++ |
| | Legal's test | - |
| Tannins and phenolic compounds | $FeCl_3$ test | ++ |
| | Dilute iodine solution test | ++ |
| | Nitric acid test | ++ |
| | Gelatin test | ++ |
| Saponins | Froth test | ++ |
| Amino acids and proteins | Biuret's test | - |
| | Millon's test | ++ |
| | Ninhydrin test | ++ |

++: Present, -: Absent, *I. kashmiriana: Iris kashmiriana*

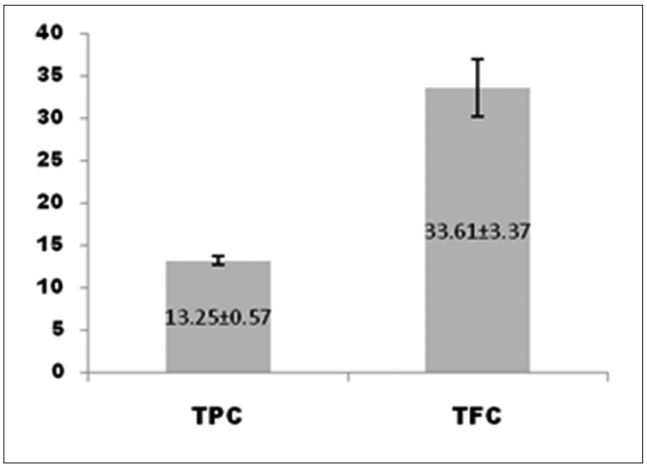


**Fig. 3: Total phenol content and total flavonoid content of *Iris kashmiriana* leaf extract**

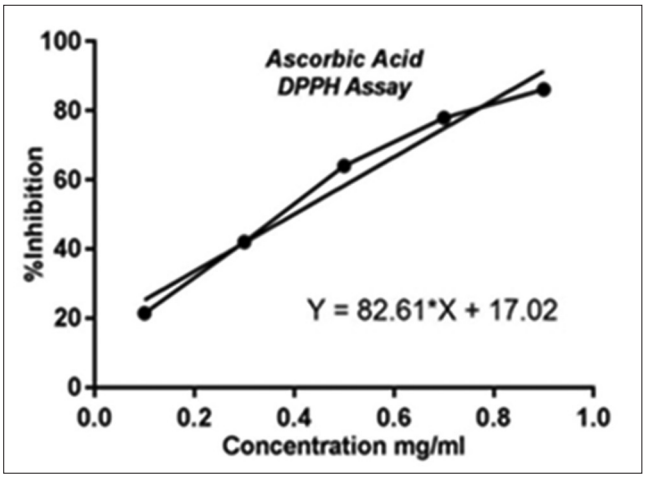


**Fig. 5: Standard ascorbic acid**

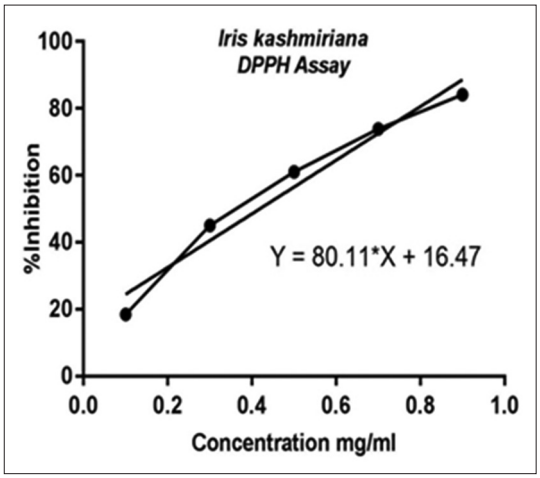


**Fig. 4: 1,1-diphenyl-2-picrylhydrazyl assay of *Iris kashmiriana* leaf extract**

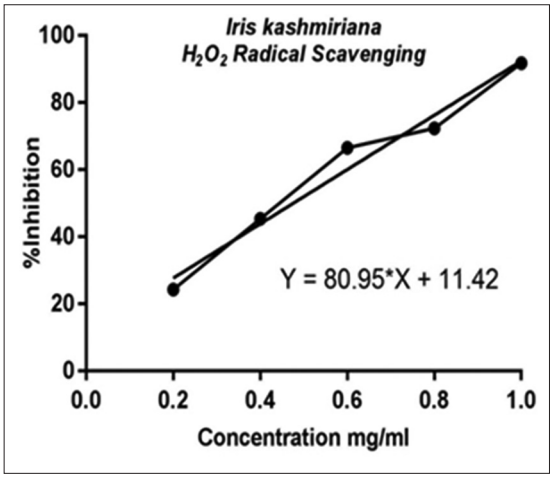


**Fig. 6: Hydrogen peroxide scavenging *by Iris kashmiriana* leaf extract**

**Table 2: The reducing power of *I. kashmiriana***

| S. No. | Concentration (µg/ml) | Absorbance (nm) |
|---|---|---|
| 1 | 100 | 0.229 |
| 2 | 200 | 0.355 |
| 3 | 300 | 0.534 |
| 4 | 400 | 0.689 |
| 5 | 500 | 0.755 |

such as these ingredients possess antioxidant, antifungal, anticancer, and antimicrobial activities [16,17]. Phenolic and flavonoid compounds protect plant and plant parts from oxidative damage and thus provide a better antioxidant option for human beings [18].

Ethanolic leaf extract of *I. kashmiriana* showed the presence of bioactive compounds and possessed remarkable amount of phenolics and flavonoids. Our results revealed that *I. kashmiriana* extract acquires potential antioxidant activity by donating hydrogen to DPPH (deep violet color) free radical and converted it into α,α- diphenyl-β- picrylhydrazine (colorless). The antioxidant potential is indicated by discoloration [19]. Antioxidant activity is directly related with the amount of phenols present in extract [20]. It has been indicated that phenolic compounds are directly involved in the scavenging activity of free radicals [21,22].

$H_2O_2$, a weak oxidizing agent, inactivates various enzymes by oxidizing their thiol (–SH) group. However, sometimes it gives rise to very toxic hydroxyl free radical, which is very harmful and lethal to the cells [23]. Free hydroxyl radical is formed by interaction of $H_2O_2$ with $Fe^{2+}$ and $Cu^{2+}$ inside cell [24]. *I. kashmiriana* extract showed dose-dependent scavenging activity of $H_2O_2$ as stated earlier in the results. Furthermore, the ferric reducing power showed the extract acts as a good reductant as the extract reduces ferric ion of ferric cyanide to ferrous ion (Perl's Prussian blue). The reducing property of extract or compound depends on the reductants [25]. Similar results were found in *Combretum albidum* [26] and *Piper umbellatum* [27] leave extracts. In this study, the results clearly indicated that our plant extract exhibit a significant and potential antioxidant activity.

## CONCLUSION

The demand for natural antioxidants is increasing day by day. Our results showed *I. kashmiriana* contained an excellent quantity of phenolics and flavonoids which bestow antioxidant property to the plant. Hence, the plant can be refined for such chemical compounds to cure different diseases and ailments with no or less side effects.

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