SUPPLEMENTARY MATERIAL

Phenolic, flavonoid contents, anticholinesterase and antioxidant evaluation of *Iris germanica var; florentina*

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**Abstract**

This study was designed to investigate antioxidant and anticholinesterase potential of *Iris germanica var; florentina* (IGF). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory potential of plant samples were investigated by Ellman's assay. Antioxidant activity was performed using DPPH, \( \text{H}_2\text{O}_2 \) and ABTS free radical scavenging assays. Total phenolics and flavonoids contents were expressed in mg GAE/g dry weight and mg RTE/g respectively. In AChE inhibition assay, Ig.Fl, Ig.Sp and Ig.Cf fractions exhibited highest activity with IC\(_{50}\) values of < 0.1, 5.64 and 19 µg/ml respectively. In BChE inhibitory assay, Ig.Fl, Ig.Sp, Ig.Cf and Ig.Cr were most active with IC\(_{50}\) of < 0.1, < 0.1, 31 and 78 µg/ml respectively. In DPPH assay, Ig.Fl and Ig.Cf exhibited highest inhibition of free radicals, 80.52% (IC\(_{50}\) = 9 µg/ml) and 78.30% (IC\(_{50}\) = 8 µg/ml) respectively. In ABTS assay Ig.Cr, Ig.Cf, Ig.Fl and Ig.Sp exhibited IC\(_{50}\) values of < 0.1, 2, 2 and 3 µg/ml respectively.

**Key words:** Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), DPPH, \( \text{H}_2\text{O}_2 \), ABTS, Ellman's assay and *Iris germanica*

4. Experimental

4.1. Collection of plant material and extraction

The rhizome of IGF was collected from district Malakand, Khyber PakhtoonKhwa, Pakistan in August 2012. The plant name was confirmed by Prof. Dr. Jahandar Shah, Shaheed Benazir Bhutto University Sheringal, Dir (U), Pakistan. A sample of the dried plant material is deposited at the herbarium of the said university with voucher no (AB-1017). Rhizome was washed, shade dried and powdered. A total of 4kg powdered rhizome was soaked in 16L methanol (80%) with occasional shaking for 14days. Then the suspension obtained was filtered and concentrated.
under reduced pressure using rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) to obtain a solid mass of 300g crude methanolic extract (7.5%).

4.2. Fractionation
Fractionation was done by means of solvent-solvent extraction. *n*-Hexane, chloroform, ethyl acetate and distilled water were used to obtain the respective fractions. Distilled water was added to the crude extract and suspension was made followed by the addition of *n*-hexane (500ml) with vigorous shaking. The solution mixture was transferred into a separating funnel to make distinct layers of *n*-hexane and water. After 4h, the organic layer was separated followed by concentration under reduced pressure using rotary evaporator at 40°C. The process was repeated until purification is achieved. Same procedure was repeated to obtain Ig.Cf and Ig.EtAc. The residual aqueous portion was obtained at the end (Ayaz et al. 2014).

4.3. Extraction of crude saponins
For the extraction of saponins, powdered material (20g) of IGF rhizome was added to 100ml (20%) ethanol in a conical flask and incubated at 55°C in water bath for 4h with continuous stirring. After 4h, the mixture was filtered and re-extracted with 200ml (20%) ethanol. The solution was evaporated in a hot water bath to get volume of 40ml concentrated solution. Then it was transferred into a separating funnel and mixed with 20ml diethyl ether with continuous shaking till two layers were formed. After some time, two layers were formed, aqueous and organic layer. The aqueous layer was recovered and the organic layer was discarded. The aqueous layer was mixed with 60ml of *n*-butanol and kept for some time. The mixture was washed by adding 5% NaCl solution and was kept in a water bath at 40°C till evaporated and semisolid (1.8g) saponins (9%) were obtained (Khan et al. 2011).

4.4. Extraction of Flavonoids
The procedure of Harborne was followed for the extraction of flavonoids (Harborne 1998). Powdered plant sample weighing 20g was heated at 50°C for half an hour in 200ml of HCl (2M) under reflux. The heated mixture was then cooled and filtered using Whatman's No.42 filter paper. The filtrate was treated with equal volume of ethyl acetate. The flavonoids present in the extract were precipitated, which were recovered with the help of filter paper. The weight of flavonoids obtained was 1.5g (7.5%).
4.5. Determination of flavonoid contents

In order to determine the total flavonoid contents, the procedure of Park et al., (Park et al. 2008) was followed. Using this procedure, 0.3ml of extracts, 3.4ml of 30% methanol, 0.15ml of NaNO₂ (0.5M) and 0.15ml of AlCl₃.6H₂O (0.3M) were taken in a 10ml test tube and mixed. After 5min, 1ml of NaOH (1M) was added. The mixture was measured at 506 nm. The standard curve for the total flavonoids was made using rutin standard solution (0 to 100mg/L) under the same procedure as described above. The total flavonoids were expressed as milligrams of rutin equivalents per gram of dried sample.

4.6. Determination of Phenolic contents

The spectrophotometric method was followed for the determination of total phenolic contents (Kim et al. 2003). In brief, 1ml of the extract diluted with respective solvents was added to 9ml of deionized distilled water. The mixture was added one ml of Folin-Ciocalteu’s phenol reagent (FCR) with continuous shaking. After 5min, 10ml of 7% Na₂CO₃ solution was added and mixed well. The solution was diluted to 25ml with deionized distilled water and mixed thoroughly. After 90min at 23°C, the absorbance was measured at 750 nm using deionized distilled water as blank. The standard curve for total phenolics was made using gallic acid standard solution (0 to 100mg/L) under the same procedure as earlier described. The total phenolics were expressed as milligrams of Gallic acid equivalents (GAE) per gram of dried sample.

4.7. Anticholinesterase assays

4.7.1. Chemicals

Aquine butyrylcholinesterase (Sigma-Aldrich USA), Electric eel acetylcholinesterase (type-VI-S, Sigma-Aldrich USA), Butyrylthiocholine iodide (Sigma-Aldrich Switzerland), Acetylthiocholine Iodide (Sigma-Aldrich UK), 5,5-dithio-bis-nitrobenzoic acid (DTNB) (Sigma-Aldrich Germany), Potassium phosphate buffer (pH 8.0), Galantamine from Lycoris Sp. (Sigma-Aldrich France) were used in the study.

4.7.2. Spectroscopic analysis

The spectrophotometric method of Ellman was followed for the assessment of AChE and BChE inhibition using acetylthiocholine iodide and butyrylthiocholine iodide as substrates (Ellman et al. 1961). Briefly, in a cuvette, 5μl of AChE (0.03 U/ml) and BChE (0.01 U/ml) were taken and
205µl of plant samples (125-1000µg/ml) and DTNB (5µl) were added to the mixture followed by incubation for 15 min at 30°C. The reaction was started by adding substrates (5µl). The reaction mixture was analyzed at 412 nm by using a double beam spectrophotometer. Absorption was recorded for 4min. The appearance of yellow color indicated the formation of 5-thio-2-nitrobenzoate anion as a result of the reaction between thiocholines and DTNB. To check the non-enzymatic hydrolysis of substrate, white assay was carried out without enzymes and plant samples. The reaction mixture containing all the components excluding plant sample was taken as control. Percent enzyme activity and percent inhibition were calculated as follows;

\[ V = \frac{\Delta \text{Abs}}{\Delta t} \]

\[ \% \text{ enzyme activity} = \frac{V}{V_{\text{max}}} \times 100 \]

\[ \% \text{ enzyme inhibition} = 100 - \% \text{ enzyme activity} \]

(Where V denotes the rate of reaction in the presence of inhibitor and \( V_{\text{max}} \) denotes the rate of reaction without inhibitor)

4.8. Antioxidant activities

4.8.1. DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity

For this activity, DPPH solution was prepared by dissolving 24mg of DPPH in 100ml of methanol. The stock solutions of plant samples, each having concentration of 1mg/ml were prepared in methanol and then diluted to make concentrations of 500, 250, 125µg/ml. From each sample, 1ml solution was mixed with 1ml of DPPH solution. The solution mixture was incubated at 23°C for 30min and then the absorbance was measured at 517 nm. For positive control ascorbic acid was used (Brand-Williams et al. 1995). Experiments were performed in triplicate and the data obtained was presented as mean ± SEM. The percent radical scavenging activity was calculated using the following equation;

\[ \% \text{ scavenging} = \frac{\text{absorption of control} - \text{absorption of fraction}}{\text{absorption of control}} \times 100 \]
4.8.2. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of the samples was conducted according to the reported procedure (Ruch 1989). Solution of hydrogen peroxide (2mmol) was prepared in phosphate buffer (50mmol) having pH 7.4. Then 0.1ml of Ig.Cr, Ig.Hex, Ig.Cf, Ig.EtAc, Ig.Aq, Ig.Sp, Ig.Fl were taken in separate test tubes and their volumes were made 0.4ml by addition of phosphate buffer (50mmol) followed by the addition of 0.6ml of hydrogen peroxide and vortexed. After 10min, the absorbance of each sample was measured at 230 nm against the blank. Ascorbic acid was used as positive control and hydrogen peroxide scavenging activity was measured using the following equation;

\[ \text{H}_2\text{O}_2 \text{ scavenging activity} = \frac{1 - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \]

4.8.3. ABTS free radical scavenging assay

The antioxidant potential of Ig.Cr, Ig.Hex, Ig.Cf, Ig.EtAc, Ig.Aq, Ig.Sp and Ig.Fl of IGF was determined by using the free radicals of 2, 2-azinobis [3-ethylbenzthiazoline]-6-sulfonic acid (ABTS). ABTS (7mmol) and potassium persulfate (2.45mmol) solutions were prepared and mixed. The solution mixture was incubated for 10h in dark for production of free radicals. After incubation sufficient amount of methanol (50%) was added to adjust the absorbance of ABTS solution to 0.7 at 745 nm. Then 3ml ABTS solution was added to 300μl test sample and was transferred to a cuvette. Absorbance was measured using a double beam spectrophotometer. Ascorbic acid was taken as positive control and the data was recorded in triplicate. Percent ABTS free radicals scavenging activity was calculated as follows;

\[ \% \text{scavenging activity} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100 \]

4.9. Estimation of IC\textsubscript{50} Values

Median inhibitory values (IC\textsubscript{50}) were calculated for enzyme inhibition and antioxidant activities using Microsoft Excel program.
4.10. Statistical analysis

All the experiments were performed in triplicate and values were expressed as means ± SEM. One way ANOVA followed by multiple comparison Dunnett’s test was used for the comparison of positive control with the test groups. The P values less than 0.05 were considered as statistically significant.

4.11. Regression and linear correlation

Regression (y) and linear correlation ($R^2$) for phenolic contents and various activities (Antioxidant and anticholinesterase) were determined using Microsoft Excel 2007.

Table S1: Percent AChE and BChE inhibitory activities of crude saponins, Flavonoids, crude methanolic extract and subsequent fractions of IGF.

| Sample | Conc (μg/ml) | AChE inhibition (%) (mean±SEM) | AChE IC$_{50}$ (μg/ml) | BChE inhibition (%) (mean±SEM) | BChE IC$_{50}$ (μg/ml) |
|--------|--------------|---------------------------------|-------------------------|---------------------------------|-------------------------|
| Ig.Cr  | 3.90         | Na                              | 350                     | 19.11 ± 0.68***                 | 31.60 ± 0.83***         |
|        | 7.81         | 04.43 ± 0.29***                 | Na                      | 31.60 ± 0.83***                 | 31.60 ± 0.83***         |
|        | 15.62        | 21.44 ±0.44***                  |                         | 39.35 ± 0.41***                 | 39.35 ± 0.41***         |
|        | 31.25        | 30.81 ± 0.99***                 |                         | 43.68 ± 0.14***                 | 43.68 ± 0.14***         |
|        | 62.50        | 38.11 ± 0.68***                 | 350                     | 44.74 ± 0.74***                 | 44.74 ± 0.74***         |
|        | 125          | 44.72 ± 0.19***                 |                         | 63.78 ± 0.08***                 | 63.78 ± 0.08***         |
|        | 250          | 48.63 ± 0.13***                 |                         | 66.18 ± 0.08***                 | 66.18 ± 0.08***         |
|        | 500          | 51.54 ± 0.21***                 |                         | 68.51 ± 0.18***                 | 68.51 ± 0.18***         |
|        | 1000         | 55.50 ± 0.42***                 |                         | 71.06 ± 1.91***                 | 71.06 ± 1.91***         |
| Ig.Hex | 3.90         | Na                              | 710                     | 29.32 ± 1.42***                 | 29.32 ± 1.42***         |
|        | 7.81         | Na                              |                         | 42.72 ± 0.09***                 | 42.72 ± 0.09***         |
|        | 15.62        | 11.43 ± 0.29***                 |                         | 44.31 ± 1.25***                 | 44.31 ± 1.25***         |
|        | 31.25        | 19.15 ± 0.83***                 |                         | 47.41 ± 0.17***                 | 47.41 ± 0.17***         |
|        | 62.50        | 33.44 ± 0.44***                 |                         | 49.47 ± 0.22***                 | 49.47 ± 0.22***         |
|        | 125          | 40.79 ± 0.73***                 |                         | 51.72 ± 0.30***                 | 51.72 ± 0.30***         |
|        | 250          | 44.60 ± 0.83***                 |                         | 52.43 ± 1.09***                 | 52.43 ± 1.09***         |
|        | 500          | 47.75 ± 0.75***                 |                         | 52.43 ± 1.09***                 | 52.43 ± 1.09***         |
|        | 1000         | 51.72 ± 0.30***                 |                         | 64.56 ± 0.20***                 | 64.56 ± 0.20***         |
| Ig.Cf  | 3.90         | 31.73 ± 0.48***                 | 19                      | 35.47 ± 0.47***                 | 35.47 ± 0.47***         |
|        | 7.81         | 33.25 ± 0.68***                 |                         | 39.75 ± 0.75***                 | 39.75 ± 0.75***         |
|        | 15.62        | 49.93 ± 1.04***                 |                         | 44.72 ± 0.19***                 | 44.72 ± 0.19***         |
|        | 31.25        | 54.35 ± 0.91***                 |                         | 51.18 ± 0.18***                 | 51.18 ± 0.18***         |
|        | 62.50        | 61.56 ± 1.53***                 |                         | 52.43 ± 1.09***                 | 52.43 ± 1.09***         |
|        | 125          | 63.68 ± 0.14***                 | 19                      | 64.56 ± 0.20***                 | 64.56 ± 0.20***         |
|        | 250          | 66.66 ± 0.82***                 |                         | 68.58 ± 0.21***                 | 68.58 ± 0.21***         |
|        | 500          | 69.35 ± 0.41***                 |                         | 70.57 ± 1.81***                 | 70.57 ± 1.81***         |
|        | 1000         | 71.43 ± 0.61***                 |                         | 73.56 ± 1.15***                 | 73.56 ± 1.15***         |
|                | Galanthamine | Ig.Fl | Ig.Sp | Ig.Aq | Ig.EtAc |
|----------------|--------------|-------|-------|-------|---------|
| **Concentration (μM)** | **3.90** | **7.81** | **15.62** | **31.25** | **62.50** |
| **500** | 68.35 ± 0.14*** | 53.63 ± 0.85*** | 59.41 ± 0.21** | 52.20 ± 0.70*** | 37.70 ± 0.29 **|
| **1000** | 67.53 ± 0.57*** | 56.22 ± 1.03*** | 63.55 ± 0.43** | 54.22 ± 0.81*** | 29.18 ± 0.67 |
| **5000** | 66.48 ± 0.23*** | 57.33 ± 0.09*** | 66.98 ± 0.67** | 57.31 ± 0.03* | 12.43 ± 0.55*** |
| **10000** | 65.61 ± 1.66*** | 61.63 ± 0.15*** | 66.14 ± 0.51*** | 61.63 ± 0.15*** | 20.22 ± 1.15*** |

The data is represented as mean±SEM, (n=3). Values significantly different as compare to positive control, *:P < 0.05, **:P < 0.01, ***: P < 0.001
Table S2: Percent DPPH scavenging activity of flavonoids, crude methanolic and various fractions of IGF.

| Samples | Concentrations (µg/ml) | Scavenging (%) | IC$_{50}$ (µg/ml) |
|---------|------------------------|----------------|-------------------|
| Ig.Cr   | 125                    | 45.57 ± 0.23*  | 150               |
|         | 250                    | 62.77 ± 0.95*  |                   |
|         | 500                    | 73.36 ± 0.45*  |                   |
| Ig.Hex  | 125                    | 42.74 ± 0.59** | 198               |
|         | 250                    | 55.65 ± 0.22** |                   |
|         | 500                    | 61.67 ± 0.19** |                   |
| Ig.Cf   | 125                    | 69.34 ± 0.06ns |                   |
|         | 250                    | 74.42 ± 0.22ns | 08                |
|         | 500                    | 78.30 ± 0.53ns |                   |
| Ig.EtAc | 125                    | 61.65 ± 0.06ns |                   |
|         | 250                    | 70.88 ± 0.11ns | 31                |
|         | 500                    | 74.20 ± 0.67ns |                   |
| Ig.Aq   | 125                    | 41.86 ± 0.08***|                   |
|         | 250                    | 53.49 ± 0.22***| 223               |
|         | 500                    | 58.92 ± 0.28***|                   |
| Ig.Fl   | 125                    | 70.35 ± 0.21ns |                   |
|         | 250                    | 78.30 ± 0.53ns | 09                |
|         | 500                    | 80.52 ± 0.30ns |                   |
| Ig.Sp   | 125                    | 64.89 ± 1.70ns |                   |
|         | 250                    | 72.55 ± 1.75ns | 30                |
|         | 500                    | 79.43 ± 0.52ns |                   |
| Ascorbic acid | 125        | 73.73 ± 0.15  | 14                |
|         | 250                    | 84.61 ± 2.31  |                   |
|         | 500                    | 89.49 ± 0.16  |                   |

Values significantly different as compare to positive control, *: P < 0.05, **: P < 0.01, ***: P < 0.001
Figure S1: Total phenolics in different fractions of IGF. Values expressed as mg GAE/g of dry sample.

Figure S2: Total flavonoids in different fractions of IGF. Values expressed as mg RTE/g of dry sample.
**Figure S3:** H$_2$O$_2$ scavenging activity of flavonoids, crude methanolic and various fractions of IGF.

Results of Dunnett’s multiple comparison test applied to H$_2$O$_2$ scavenging activity. Values significantly different as compare to positive control, *: P < 0.05, **: P < 0.01, ***: P < 0.001. ns: not significantly different in comparison to positive control.

**Figure S4:** Results of ABTS free radical scavenging scavenging activity of flavonoids, crude methanolic and various fractions of IGF.
Results of Dunnett’s multiple comparison test applied to ABTS scavenging activity. Values significantly different as compare to positive control, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. ns: not significantly different in comparison to positive control.
\[ y = 0.315x + 38.57 \]
\[ R^2 = 0.640 \]

\[ y = 0.262x + 48.73 \]
\[ R^2 = 0.547 \]

\[ y = 0.300x + 43.97 \]
\[ R^2 = 0.773 \]

\[ y = 0.314x + 42.49 \]
\[ R^2 = 0.864 \]

\[ y = 0.300x + 43.97 \]
\[ R^2 = 0.773 \]

\[ y = 0.314x + 42.49 \]
\[ R^2 = 0.864 \]
Figure S5 (A, B, C, D, E): Linear correlation between phenolic contents and AChE inhibitory activity (A), BChE inhibitory activity (B), DPPH (C), H$_2$O$_2$ (D), and ABTS scavenging activity (E).

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