SUPPLEMENTARY MATERIAL

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

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Abstract

This study was designed to investigate antioxidant and anticholinestrase potential of *Iris germanica* var; *florentina* (IGF). Acetylcholinestrase (AChE) and butyrylcholinestrase (BChE) inhibitory potential of plant samples were investigated by Ellman's assay. Antioxidant activity was performed using DPPH, H₂O₂ 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.FI, Ig.Sp and Ig.Cf fractions exhibited highest activity with IC₅₀ values of **< 0.1, 5.64 and 19** µg/ml respectively. In BChE inhibitory assay, Ig.FI, Ig.Sp, Ig.Cf and Ig.Cr were most active with IC₅₀ of **< 0.1, < 0.1, 31 and 78** µg/ml respectively. In DPPH assay, Ig.FI and Ig.Cf exhibited highest inhibition of free radicals, 80.52% (IC₅₀ = 9 µg/ml) and 78.30% (IC₅₀ = 8 µg/ml) respectively. In ABTS assay Ig.Cr, Ig.Cf, Ig.FI and Ig.Sp exhibited IC₅₀ values of **< 0.1, 2, 2 and 3** µg/ml respectively.

Key words: Acetylcholinestrase (AChE), butyrylcholinestrase (BChE), DPPH, H₂O₂, 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 NaNO_2 (0.5M) and 0.15ml of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (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% Na_2CO_3 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;

$$V = \frac{\Delta \text{Abs}}{\Delta t}$$

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

$$\% \text{ enzyme inhibition} = 100 - \% \text{ enzyme activity}$$

(Where V denotes the rate of reaction in the presence of inhibitor and $V_{\max}$ 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.FI 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.FI 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 IC₅₀ Values

Median inhibitory values (IC₅₀) 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 $\pm$ 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 anticholinestrase) 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 ($\mu\text{g/ml}$)	AChE inhibition (%) (mean $\pm$ SEM)	AChE IC ₅₀ ($\mu\text{g/ml}$)	BChE inhibition (%) (mean $\pm$ SEM)	BChE IC ₅₀ ($\mu\text{g/ml}$)
Ig.Cr	3.90	Na	350	19.11 $\pm$ 0.68***	78
	7.81	04.43 $\pm$ 0.29***		31.60 $\pm$ 0.83***	
	15.62	21.44 $\pm$ 0.44***		39.35 $\pm$ 0.41***	
	31.25	30.81 $\pm$ 0.99***		43.68 $\pm$ 0.14***	
	62.50	38.11 $\pm$ 0.68***		44.74 $\pm$ 0.74***	
	125	44.72 $\pm$ 0.19***		63.78 $\pm$ 0.08***	
	250	48.63 $\pm$ 0.13***		66.18 $\pm$ 0.08***	
	500	51.54 $\pm$ 0.21***		68.51 $\pm$ 0.18***	
	1000	55.50 $\pm$ 0.42***		71.06 $\pm$ 1.91***	
Ig.Hex	3.90	Na	710	Na	1170
	7.81	Na		06.14 $\pm$ 0.59***	
	15.62	11.43 $\pm$ 0.29***		14.77 $\pm$ 1.15***	
	31.25	19.15 $\pm$ 0.83***		27.76 $\pm$ 0.81***	
	62.50	33.44 $\pm$ 0.44***		29.32 $\pm$ 1.42***	
	125	40.79 $\pm$ 0.73***		42.72 $\pm$ 0.09***	
	250	44.60 $\pm$ 0.83***		44.31 $\pm$ 1.25***	
	500	47.75 $\pm$ 0.75***		47.41 $\pm$ 0.17***	
	1000	51.72 $\pm$ 0.30***		49.47 $\pm$ 0.22***	
Ig.Cf	3.90	31.73 $\pm$ 0.48***	19	35.47 $\pm$ 0.47***	31
	7.81	33.25 $\pm$ 0.68***		39.75 $\pm$ 0.75***	
	15.62	49.93 $\pm$ 1.04***		44.72 $\pm$ 0.19***	
	31.25	54.35 $\pm$ 0.91***		51.18 $\pm$ 0.18***	
	62.50	61.56 $\pm$ 1.53***		52.43 $\pm$ 1.09***	
	125	63.68 $\pm$ 0.14***		64.56 $\pm$ 0.20***	
	250	66.69 $\pm$ 0.82***		68.58 $\pm$ 0.21***	
	500	69.35 $\pm$ 0.41***		70.57 $\pm$ 1.81***	
	1000	71.43 $\pm$ 0.61***		73.56 $\pm$ 1.15***	

Ig.EtAc	3.90	37.70 ± 0.29		29.18 ± 0.67	
	7.81	41.99 ± 0.87***		36.35 ± 0.41***	
	15.62	46.55 ± 1.39***		39.65 ± 0.94***	
	31.25	49.68 ± 0.14***		44.69 ± 0.82***	
	62.50	51.45 ± 0.41***	43	48.55 ± 1.63***	74
	125	61.63 ± 0.41***		60.59 ± 0.23***	
	250	63.68 ± 0.83***		62.70 ± 0.55***	
	500	66.70 ± 0.14***		65.61 ± 1.66***	
	1000	68.35 ± 0.14***		67.53 ± 0.57***	
Ig.Aq	3.90	Na		12.43 ± 0.55***	
	7.81	Na		20.22 ± 1.15***	
	15.62	09.67 ± 0.12***		27.40 ± 0.62***	
	31.25	14.11 ± 1.83***		35.59 ± 1.19***	
	62.50	18.55 ± 1.43***	1162	39.67 ± 0.54***	83
	125	31.60 ± 0.03***		54.55 ± 0.19***	
	250	38.39 ± 0.14***		59.54 ± 0.50***	
	500	44.75 ± 0.59***		61.63 ± 0.15***	
	1000	47.41 ± 0.21***		66.48 ± 0.23***	
Ig.Sp	3.90	48.26 ± 1.25***		52.20 ± 0.70***	
	7.81	53.59 ± 0.82***		54.22 ± 0.81***	
	15.62	56.35 ± 1.12***		57.33 ± 0.09***	
	31.25	61.54 ± 0.78***	5.64	63.08 ± 0.24 **	<0.1
	62.50	66.14 ± 0.51***		68.54 ± 1.13**	
	125	70.37 ± 0.21***		71.48 ± 0.03*	
	250	73.75 ± 0.48***		74.53 ± 0.19*	
	500	75.32 ± 0.17***		79.31 ± 0.03*	
	1000	79.55 ± 0.49***		87.40 ± 0.16*	
Ig.Fl	3.90	59.41 ± 0.21**		53.63 ± 0.85***	
	7.81	63.55 ± 0.43**		56.22 ± 1.03***	
	15.62	66.98 ± 0.67**		60.38 ± 0.52***	
	31.25	68.35 ± 1.13**		64.11 ± 0.94**	
	62.50	71.44 ± 0.72**	<0.1	69.43 ± 0.61**	<0.1
	125	74.20 ± 0.67 *		73.58 ± 0.21 **	
	250	77.56 ± 1.15 *		77.64 ± 0.22 *	
	500	80.50 ± 0.57 *		81.56 ± 0.13 *	
	1000	83.14 ± 0.19 *		89.34 ± 1.15 *	
Galanthamine	3.90	63.10 ± 0.66		68.14 ± 1.03	
	7.81	69.96 ± 0.76		70.11 ± 1.02	
	15.62	76.34 ± 1.09		71.36 ± 0.63	
	31.25	77.35 ± 0.64		76.18 ± 0.71	
	62.50	79.43 ± 1.81	<0.1	81.69 ± 0.27	<0.1
	125	83.50 ± 0.19		83.55 ± 0.16	
	250	87.76 ± 0.07		87.27 ± 0.07	
	500	93.05 ± 1.41		93.58 ± 0.18	
	1000	95.36 ± 0.31		95.66 ± 0.22	

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 ($\mu\text{g/ml}$)	Scavenging (%)	IC ₅₀ ($\mu\text{g/ml}$)
Ig.Cr	125	45.57 $\pm$ 0.23*	150
	250	62.77 $\pm$ 0.95*	
	500	73.36 $\pm$ 0.45*	
Ig.Hex	125	42.74 $\pm$ 0.59**	198
	250	55.65 $\pm$ 0.22**	
	500	61.67 $\pm$ 0.19**	
Ig.Cf	125	69.34 $\pm$ 0.06 ^{ns}	08
	250	74.42 $\pm$ 0.22 ^{ns}	
	500	78.30 $\pm$ 0.53 ^{ns}	
Ig.EtAc	125	61.65 $\pm$ 0.06 ^{ns}	31
	250	70.88 $\pm$ 0.11 ^{ns}	
	500	74.20 $\pm$ 0.67 ^{ns}	
Ig.Aq	125	41.86 $\pm$ 0.08**	223
	250	53.49 $\pm$ 0.22**	
	500	58.92 $\pm$ 0.28**	
Ig.Fl	125	70.35 $\pm$ 0.21 ^{ns}	09
	250	78.30 $\pm$ 0.53 ^{ns}	
	500	80.52 $\pm$ 0.30 ^{ns}	
Ig.Sp	125	64.89 $\pm$ 1.70 ^{ns}	30
	250	72.55 $\pm$ 1.75 ^{ns}	
	500	79.43 $\pm$ 0.52 ^{ns}	
Ascorbic acid	125	73.73 $\pm$ 0.15	14
	250	84.61 $\pm$ 2.31	
	500	89.49 $\pm$ 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: H₂O₂ scavenging activity of flavonoids, crude methanolic and various fractions of IGF.

Results of Dunnett's multiple comparison test applied to H₂O₂ 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 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.

Figure S5 (A, B, C, D, E): Linear correlation between phenolic contents and AChE inhibitory activity (A), BChE inhibitory activity (B), DPPH (C), H₂O₂ (D), and ABTS scavenging activity (E).

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