Phenolic, Flavonoid Content and Radical Scavenging Activity of *Smilax china* with its Inhibitory Potential against Clinically Important Enzymes

Hafiz Ibtesam Ahmad[a], Haji Muhammad Shoaib Khan[a]*, Shakeel Ijaz[ab] Naveed Akhtar[a]

[a]Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Punjab, Pakistan.

[b]School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, University of Dublin, Dublin 2, Ireland

*Corresponding Author

Haji Muhammad Shoaib Khan

Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur 63100, Punjab, Pakistan

Email: shoaib.khan@iub.edu.com

Telephone # +923311143022

FAX# +92622883121

Abstract

Smilax china (*SC*) is a medicinal plant that has been traditionally used for a number of pathological disorders. In current study, its various fractions were assessed for radical scavenging, phenolic, flavonoid content and enzyme inhibition. The methanolic extract (MSC) of *SC* was subjected to fractionation using different solvents including *n*-hexane, benzene, chloroform, ethyl acetate and *n*-butanol. Results revealed that ethyl acetate fraction showed maximum phenolic (101.81±0.13mg GAE/g) and flavonoid (96.80±0.39mg QE/g) content with maximum radical scavenging potential (82.51±0.18%, IC_{50}=104.45 µg/ml) as well as urease (82.63±0.79%), tyrosinase (81.30±0.41%) and Butyrylcholinesterase (BChE) (62.47±0.76%) inhibition at 0.5mg/ml. Whereas, maximum *α*-Glucosidase (87.56±0.13%) and Acetylcholinesterase (AChE) inhibition (82.34±0.64%) was exhibited by *n*-hexane and benzene fractions, respectively. Present study has revealed the promising radical scavenging, phenolic, flavonoid and enzyme inhibitory potential of various fractions of *SC* extract. Thus, the study is a step forward towards evidence-based phyto-medicine.

Key words: *Smilax china*, fractionation, enzyme inhibition, radical scavenging activity

Experimental

1.1. Material

DPPH, gallic acid, quercetin, aluminum chloride, sodium nitrite, yeast α-glucosidase, electric eel acetylcholinesterase, equine butyrylcholinesterase, jack bean urease, L-tyrosine, arbutin, lyophilized mushroom tyrosinase and their substrates and inhibitors were purchased from Sigma-Aldrich (Germany). Folin-Ciocalteu reagent (FCR), HPLC-grade methanol, ethyl acetate, chloroform, benzene and n-hexane were purchased from Merck (Darmstadt, Germany).

1.2. Plant material

Shade dried, disease free roots of *SC* were obtained from an herbal drug store at Bahawalpur and identified from the Department of Life Sciences, The Islamia University of Bahawalpur, Bahawalpur (Voucher Number 66/LS-16/X/17).

1.3. Extraction and fractionation

The roots were washed with cold water to remove any foreign matter and were shade dried. These were ground into powder and passed through a sieve of number 50. 500 g of this powdered root was soaked in 800 ml methanol at room temperature for 24 hours with frequent stirring. Pressed and filtered through muslin cloth and Whatman filter paper # 42, the filtrate was subjected to evaporation under reduced pressure using rotary evaporator (at 45±1^{o}C). On evaporation a dark brown semisolid material (extract) was obtained. Its percentage yield was calculated and was stored at 4^{o}C for further use. Then the dried extract (25 g) was dispersed in double distilled water and the suspension was portioned with *n*-hexane, benzene, chloroform, ethyl acetate and *n*-butanol in increasing order of polarity consecutively. Fractions were named as MSC, *n*HSC, BSC, CSC, EASC and *n*BSC representing methanol, *n*-hexane, benzene, chloroform, ethyl acetate and *n*-butanol fraction respectively. All obtained fractions were evaporated using rotary evaporator, dried, weighed and refrigerated in sealed containers for further use.

1.4. Determination of total phenolic content

TPC was measured according to method adopted by Wolfe et al. with slight modifications using FCR (Folin-Ciocalteu's reagent) (Wolfe et al. 2003). A total volume of 200 µl was added to each well of 96 micro-well plate containing 10 µl of 10% FCR mixed with 100 µl of known concentration of sample solution. The mixture was incubated for 10 minutes and then 90 µl of

15% sodium carbonate solution was added to it. Resultant mixture was again incubated for 1.5 hours at 37ºC. Absorbance was measured at 750 nm by using LT-4500 96-well microplate reader (Labtech, UK). Positive control (gallic acid) and negative controls (blank) were used for reference. TPC was calculated using the calibration curve (ranging from 0-100 µg of gallic acid). Data was expressed as milligram gallic acid equivalent per gram of extract (mg of GAE/g E).

1.5. Determination of total flavonoid content

Total flavonoid content (TFC) of various fractions was measured using modified colorimetric method (Wolfe et al. 2003). A total of 200 µl, containing 100 µl of sample solution and 25 µl of 1% sodium nitrate solution allowed to stand for 5 min. After adding 10 µl of 10% aluminum chloride, this mixture was given 5 minutes reaction time. Then, 35 µl of 4% sodium hydroxide solution was added and the resultant mixture was diluted using 30 µl of methanol. Absorbance was measured at 510 nm, all readings were taken as triplicate and their mean value was calculated. Quercetin was used as positive control and to develop the calibration curve.

1.6. Antioxidant activity

DPPH method was used to determine the antioxidant activity according to protocol devised by Shamaida et al. (Shimada et al. 1992). 100 µl of mixture containing 90 µl of DPPH solution (0.3mM) and 10 µl of sample solution was incubated for 30 min in dark at room temp. Absorbance was measured at 510 nm, all readings were taken as triplicate and their mean value was calculated. Both positive control (gallic acid and quercetin) and negative control (blank) were used. Percentage inhibition was measured using following equation;

$$Inhibiton\ (\%) = \left(\frac{Abs.of\ blank - Abs.of\ sample}{Abs.of\ blank}\right) \times 100$$

IC_{50} values were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

1.7. Enzymatic assay

1.7.1. α-Glucosidase inhibition assay

A modified method devised by Liu et al. was adopted to evaluate α-glucosidase activity (Liu et al. 2014). A total volume of 100 µl was prepared containing 70 µl of phosphate buffer (50mM, pH 6.8), 10 µl of each sample solution and yeast α-glucosidase enzyme (0.0234 units). This mixture was incubated at 37ºC for 10 min and pre-read at 400 nm. Then, 10 µl of *p*-nitrophenyl glucopyranoside (substrate, 0.5mM) was added to this reaction mixture and was incubated at 37ºC for 30 min. Absorbance was measured at 400 nm, all readings were taken as triplicate and their mean value was calculated. Enzyme inhibition was measured by following equation;

$$Inhibiton\ (\%) = \left(\frac{Abs.of\ control - Abs.of\ sample}{Abs.of\ control}\right) \times 100$$

1.7.2. AChE and BChE inhibition assay

For this assay a Ellman's modified colorimetric method was adopted (Ellman et al. 1961). 100 µl of reaction mixture was prepared containing 60 µl of phosphate buffer, 10 µl of both sample solution and enzyme. Then it was pre-read at 405 nm. Incubated for 10 min at 37ºC and then 10 µl of acetylcholine iodide (substrate, 0.5mM) was added followed by the addition of 10 µl of DTNB (Elman's reagent, 0.5mM). This mixture was again incubated at 37ºC for 15 min and absorbance was measured at 405 nm. Enzyme inhibition was calculated using above mentioned equation.

1.7.3. Urease inhibition assay

A modified method of Weatherburn was adopted for this assay (Weatherburn 1967). 200 µl volume containing 10 µl of each phosphate buffer and test compound and 25 µl of jack bean urease (0.015 units) was used in this assay. This reaction mixture was incubated at 37ºC for 10 min followed by the addition of 40 µl of urea (substrate, 20mM). This mixture was again incubated at 37ºC for 10 min and pre-read at 625nm. Then, 115 µl of phenol hypochlorite reagent was added to it and incubated at 37ºC for 10 min. Absorbance was measured at 540nm and enzyme inhibition was calculated using above mentioned equation.

1.7.4. Tyrosinase inhibition assay

Liang's modified method was used to evaluate tyrosinase inhibition activity in present study (Liang et al. 2012). Briefly, 100 µl of 5 mM L-tyrosine, 20 µl of 0.1 M phosphate buffer (pH 6.8) and 40 µl of test solution was prepared. Then, 40 µl of mushroom tyrosinase (200 units/ml) solution in the same buffer was added and incubated at 37 ºC for about 20 min. Absorbance was measured at 450 nm and enzyme inhibition was calculated using above mentioned equation.

1.7.5. Statistical analysis

All the readings were taken in triplicate and expressed as mean with standard deviation. Statistical analysis was done by using one way ANOVA with $p<0.05$ as significant difference between and within groups.

References

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Table and Figures

Table S1 Extraction yield and antioxidant activity of various fractions of *SC*

Fraction	Yield g (%)	Antioxidant Activity	
		% Inhibition (1mg/ml)	IC_{50}(µg/ml)
MSC	40 (8)	79.33 ± 0.15^{a}	185.03 ± 0.29
n-HSC	1.96 (0.392)	48.98 ± 0.07^{b}	> 1000
BSC	0.94 (0.188)	52.96 ± 0.58^{b}	628.99 ± 0.21
CSC	1.3 (0.26)	67.55 ± 0.26^{c}	233.69 ± 0.34
EASC	0.78 (0.156)	82.51 ± 0.18^{a}	104.45 ± 0.18
n-BSC	1.91 (0.382)	59.26 ± 0.13^{d}	458.12 ± 0.37
Gallic Acid	-	84.00 ± 0.16^{a}	50.23 ± 0.68
Quercetin	-	83.14 ± 0.25^{a}	98.42 ± 1.01

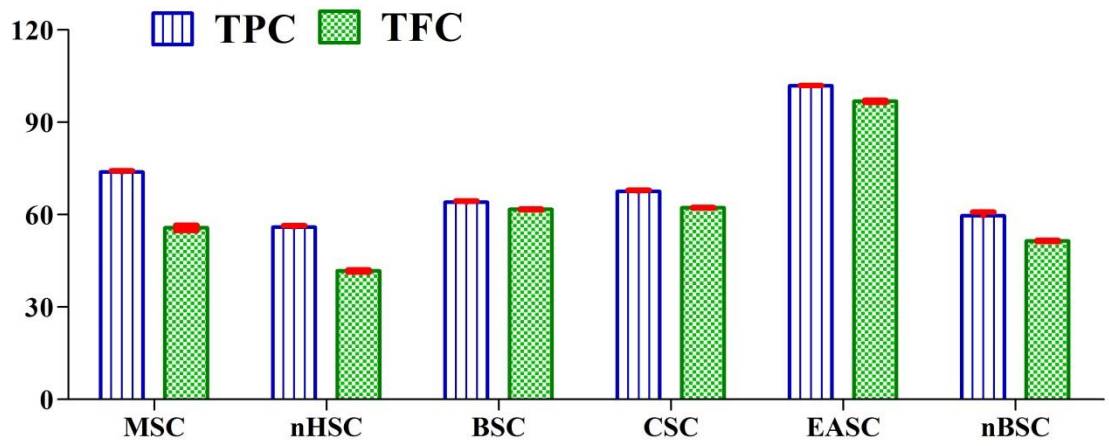
All readings were measured as triplicate and their mean value was taken. IC_{50} (µg/ml) concentration for scavenging 50% of DPPH radicals. Superscripts^{a-e} are significantly different from each other

Legends to the Figures

Legends to Figure S**1:** Total phenolic and total flavonoid content of various fractions of *SC* extract

Legends to Figure S2: Enzyme inhibitory activity of various fractions of *SC* extract **(A)** *α*-Glucosidase inhibition **(B)** AChE and BChE inhibition **(C)** Urease inhibition **(D)** Tyrosinase inhibition

Figure S1.


TPC
TFC
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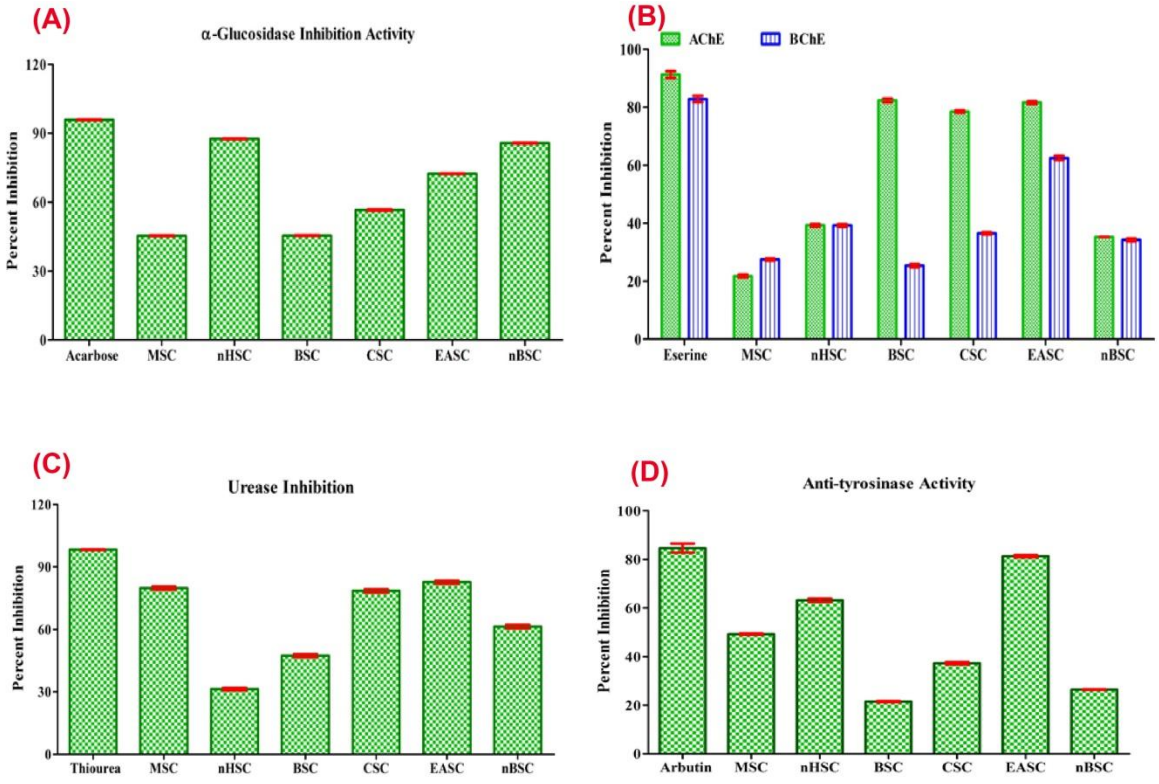
90
60
30
0
MSC
nHSC
BSC
CSC
EASC
nBSC

Figure S2.


(A)
α-Glucosidase Inhibition Activity
Percent Inhibition
Acarbose
MSC
nHSC
BSC
CSC
EASC
nBSC
(B)
AChE
BChE
Percent Inhibition
Eserine
(C)
Urease Inhibition
Thiourea
(D)
Anti-tyrosinase Activity
Arbutin