Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 568-570
Received: 01-07-2017
Accepted: 02-08-2017

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Anti-oxidant Constituents from *Corydalis govaniana* Wall and *C. casimiriana* Duthie and Prain ex Prain

Ram Lal Shrestha and Achyut Adhikari

Abstract
Corydalis govaniana Wall. and *C. casimiriana* Duthie and Prain ex Prain., have been used in the treatment of syphilis, scrofula, cutaneous infections, along with diarrhea and dysentery. One tetrahydro-protoberberine type alkaloid, govaniadine (1); and six other alkaloids, caseadine (2), caseamine (3), protopine (4), stylopine (5), apocavidine (6), and fagarine I (7) were isolated from these two medicinal plants. Caseamine showed the best anti-oxidant (DPPH radical scavenging) activity (IC_{50} = 40.8 ± 0.9 µM). Govaniadine and caseadine showed moderate antioxidant activity (IC_{50} = 83.7 ± 1.7 µM), and IC_{50} = 75.7 ± 0.9 µM, respectively).

Keywords: Corydalis, caseamine, govaniadine, antioxidant activity, alkaloids

Introduction
Corydalis govaniana Wall. and *C. casimiriana* Duthie and Prain ex Prain are the important herb and has been used to cure scrofula, syphilis, diarrhea and dysentery. Also, secondary metabolite of these plant has been showing inhibitory effect against hepatitis virus, amoeba, tumors, liver cancer, as well as acesodyne and sedative, improved immunological function, hepatocirrhosis, ascites, etc. [1]. The excellent bioactivity profile and ethno-botanical uses of these plants attract us to isolate fully characterized pure compounds and for bioassay screening of these compounds.

Reactive oxygen species (ROS) with oxidative stress can react with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, leading to atherosclerosis, arthritis, cardiovascular disease, emphysema, cirrhosis, arteriosclerosis, Alzheimer's disease [2,3,4]. Excess level of iron Fe (II) increases the H_2O_2 conversion to hydroxyl radical ($OH^{\bullet}$) as well as amplifies ROS generation. Therefore, scavengers of free radical and iron chelators are becoming increasingly important to control and prevent the excessive ROS production. Iron chelating agents emerged as effective secondary antioxidants as they stabilize the oxidized state of metal ion by reducing their redox potential [5,6]. Nitric oxide radical, trigger some biological functions including vascular homeostasis, antitumor, antimicrobial, neurotransmission etc. despite of having a beneficial role it has contribution in oxidative stress as well, nitric oxide radical on reacting with superoxide forms peroxinitrite anion, it can also generate hydroxyl radical on decomposition. Inhibition of nitric oxide radical therefore could have antioxidant potential [7]. ROS generation in cells is reduced by either inhibiting generation of superoxide anion, or by the dismutation of superoxide anion into oxygen and peroxide [8]. Antioxidants counteract the oxidative stress due to ROS before they attack cells. There are several nutrients in food and plant extracts that contain antioxidants [9].

Materials and Methods
Plant Collection and Extraction
The whole plant of both *C. govaniana* and *C. casimiriana* were collected from Langtang, Rasuwa, Nepal, and identified by Mr. Sanjiv Kumar Rai, Taxonomist, Department of Plant Resources, Thapathali, Kathmandu, Nepal. A voucher specimen, CG-207, has been deposited in Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Air-dried whole plant powder was soaked and extracted with methanol. After evaporation under reduced pressures, the residue was stirred with 7% citric acid for five hours and filtered and neutralized with ammonia solution and extracted with chloroform. The chloroform extract was subjected to column chromatography over silica-gel column by using acetone/hexanes with a few drops of diethylamine with increasing polarity, which afforded the compounds 1-7.

Structure elucidation of pure compounds: Details of structure elucidation of compounds 1-4has already published in our previous paper [1]. Structure of compounds 5-7 were deduced from different mass and NMR techniques. All the physical and spectral data of compounds 5-7 were found to be similar with reported compounds from same genus. [10, 11]

DPPH free radical scavenging assay

The free radical scavenging capacity of the compounds was measured by evaluating the scavenging activities of the stable 1,1-diphenyl 2-picrylhydrazyl (DPPH) [12]. Test compounds were allowed to react with stable 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH prepared in ethanol at final concentration of 300 mM) for half an hour at 37 °C. After incubation, the decrease in absorption was measured at 515 nm using multi-plate reader (Spectra Max-384, Molecular Device, USA). DMSO was taken as control.

***In vitro* Measurement of Nitric Oxide Radical Scavenging Ability**

The NO scavenging ability was determined according to the modified method of Hertog [13]. The reaction mixture containing 12 µL of 1.0 mM of test sample (in DMSO), 10 mM of potassium phosphate buffer at pH 7.4 and 10 mM of sodium nitropruside, was incubated at 25°C for 150 min for the formation of nitrite ions. After incubation, 50 µL of sulphanalic acid reagent (0.33% in 20% glacial acetic acid) was added and allowed to stand for 5 min for completion of diazotisation. Then 0.1% w/v of N-(1-naphthyl) ethylenediaminedihydrochloride was added and stirred, and allowed to stand for 1-2 min. A pink-coloured chromophore was formed in diffused light. The absorbance of the solution was measured at 546 nm against the corresponding blank solution. Ascorbic acid and DMSO was used as the positive control and blank respectively.

***In vitro* Measurement of Iron Chelating Ability**

The iron (Fe^{2+}) chelating ability was determined according to the modified method of Koncic [6]. The concentration of Fe^{2+} ion was measured from the formation of ferrous ion–ferrozine complex. Pure compound in DMSO (500 mM) was mixed with 0.3 mM $FeCl_2$ and 0.5 mM ferrozine. Ferrozine reacted with the divalent iron to form stable violet colored complex species that were very soluble in water. The mixture was shaken and left at room temperature for 10 min. The absorbance of the resulting mixture was measured at 562 nm by microtitre plate reader (SpectraMax plus 384, Molecular Devices, CA, USA).

Statistical Analysis

All reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance) were processed by using Soft Max Pro software (Molecular Devices, CA, USA) and then by MS Excel. The % of inhibition was calculated as:
% Inhibition = 100 - (OD of test sample / OD of the control) x100

Results are presented as means ± S.E.M. as indicated in Table 1-2. IC_{50} values were determined by using EZ-FIT (enzyme kinetics software by Perrella Scientific, Inc., Amherst, USA).

Results and Discussion

In the present studies, the crude methanol extract was evaluated for DPPH anti-oxidant activity. This extract showed 83.6% RSA at 500 µg/mL and thus we went for isolation of pure compounds and further evaluated for DPPH anti-oxidant activity. All the result is presented in Table 1-2.

Table 1: DPPH radical scavenging activity of compound **1-4**

Compound No.	% DPPH radical scavenging activity [a]	IC_{50} ± S.E.M. (µM)
Govaniadine (1)	92.2	83.7 ± 1.7
Caseadine (2)	86.7	75.7 ± 0.9
Caseamine (3)	85.3	40.8 ± 0.9
Protopine (4)	79.7	156.3 ± 1.3
[b]Propylgallate	90.3	30.5 ± 0.3

S.E.M. = Standard Error of Mean at n = 3
[a]Initial screening at 500 µM
[b]Propylgallate was used as standard for radical scavenging activity

Table 2: Iron chelation and nitric oxide radical scavenging assay of compound 1-7

Compound No.	Iron chelation assay		Nitric oxide radical scavenging assay
	% inhibition at 0.5 mM	IC_{50} (µM±SEM)	% inhibition at 1.0 mM
Govaniadine (1)	79	258.1±6.4	27.5
Caseadine (2)	27	-	23.0
Caseamine (3)	72	377.6±1.6	27.9
Protopine (4)	99	178.8±2.6	16.6
Stylopine (5)	Inactive	-	42.2
Apocavidine (6)	Inactive	-	-
Fagarine I (7)	99	157.1±0.7	5.1
[a]EDTA	-	85.1±0.1	-
[b]Ascorbic acid	-	-	618.0 ±2.0

S.E.M. = Standard Error of Mean at n = 3
[a]Standard for Iron chelation assay is disodium EDTA
[b]Standard for Nitric Oxide radical scavenging assay is ascorbic acid

Caseamine exhibited the highest DPPH-RSA activity with the IC_{50} ± SEM = 40.8 ± 0.9µM, which is close the standard compound, propyl gallate (IC_{50} = 30.5 µM) we took, and followed by caseadine (IC_{50} ± SEM = 75.7 ± 0.9µM). Caseamine contains more free hydroxyl groups than caseadine, therefore it might have better ability to scavenge all the free radicals.

Acknowledgements

We thank Prof. Dr. M Iqbal Choudhary, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan for providing laboratory facility. We thank Dr. Bishnu P. Marasini for critically reading this article.

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