African Journal of Pharmacy and Pharmacology Vol. 5(16), pp. 1932-1936, 29 October, 2011
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.559
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

Screening of eleven chemical constituents from *Radix isatidis* for antiviral activity

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Accepted 07 October, 2011

This study aimed to investigate the effects of eleven chemical constituents from *Radix isatidis* (Indigowoad root) on the inhibition of a mouse lung-adapted variant of the influenza A1 virus (FM1) and respiratory syncytial virus (RSV) *in vitro* and to identify the effective antiviral components of *R. isatidis.* The inhibitory effects of these chemical constituents against FM1 and RSV were measured using the methyl thiazolyl tetrazolium (MTT) assay. Viral suppression rates were used as an evaluation index to compare the antiviral activity of the eleven chemical constituents. The results showed that the eleven chemical constituents exerted stronger inhibitory effects on FM1 as compared to RSV. Among these chemical constituents, indirubin and isovitexin had the strongest antiviral activity.

Key words: *Radix isatidis* (Indigowoad root), antiviral, activity screening.

INTRODUCTION

Respiratory syncytial virus (RSV) Indigowoad root is the dried root of Isatidis (*Isatis indigotica* Fort.), which is a family of *Cruciferae* plant. It is bitter and cooling and affects the heart and the stomach meridians. It also reduces fever, detoxifies, cools the blood and benefits the pharynx (Qing, 2009). *Radix isatidis* has multiple pharmacological properties, including anti-viral, anti-cancer, anti-bacterial and immune enhancement (Kong et al., 2008; Shin et al., 2010). Among these properties, the anti-bacterial and anti-viral functions of *R. isatidis* are the most important. *R. isatidis* contains numerous chemical components. Phenylpropanoids, organic acids and alkaloids are the three most important classes of components. Therefore, we selected individual constituents of *R. isatidis* in these three families of chemical components to identify the effective active antiviral components. The following chemical constituents were selected: organic acid, succinic acid, alkaloids (including indirubin, indigotin, 4-3(H)quinazolinone and epigoitrin), phenylpropanoids (including neohesperidin, isoliquiritigenin, isovitexin, syringin and buddleoside) and sulfur compound sinigrin.

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MATERIALS AND METHODS

Viral strains and cell lines

Respiratory syncytial virus (RSV) was obtained from the Wuhan Institute of Virology. For its amplification method, virus (0.5 ml) was inoculated to confluent cells in tissue culture flasks. Plain medium (1.5 ml) was added to the tissue culture flasks, which were shaken once every 15 min. After 1.5 h, the supernatant was removed and 4 ml of culture medium containing 2% serum was added to the flasks. After a 3 to 4 day incubation with cytopathic effects (CPE) approaching 80 to 90%, the tissue culture flasks were frozen and thawed three times. The cell suspension was collected and centrifuged at 3000 rpm for 5 min. The resulting supernatant was aliquoted and stored at -80°C until use. The mouse lung-adapted variant of influenza A1 virus (FM1) was obtained from the Department of Microbiology and Immunology at Nanjing University of Chinese Medicine. For its amplification method, the virus was inoculated into 9 to 11 days old chicken embryos and amplified twice to enhance virulence. The allantoic fluid was collected for the measurement of its hemagglutinating activity. Once the hemagglutination titer reached 1:640, the virus was aliquoted and stored at -80°C until use.

The Madin-Darby canine kidney (MDCK) epithelial cells line and the Hela cell line (human cervical cancer cells) were obtained from the Shanghai Institute of Biochemistry and Cell Biology (SIBCB), Chinese Academy of Sciences (CAS).

Drugs

Ribavirin was obtained from the Baili Pharmaceutical group in

Sichuan province (batch number: 101018). Indirubin, indigotin, isovitexin, syringin, succinic acid, sinigrin, neohesperidin, isoliquiritigenin, epigoitrin and buddleoside were purchased from the Jiangsu province food and drug administration. 4-3(H)quinazolinone was purchased from the Shanghai Shunbo Biotech. The drugs were dissolved in tissue culture medium or DMSO and subsequently mixed with tissue culture medium, filter sterilized and stored at 4°C.

Measurement of viral virulence

MDCK or Hela cells at a concentration of 1×10^5 ml^{-1} or 2×10^4 ml^{-1} were plated in 96-well tissue culture plates, respectively. Cells grew to a monolayer within 18 to 24 h, and the supernatant was discarded before use. FM1 or RSV virus was diluted to different concentrations (10^{-1}, 10^{-2}, 10^{-3}, $10^{-}4$, 10^{-5}, 10^{-6} and 10^{-7}), and 50 l of each virus concentration was added to each well in quadruplicate. Virus was adsorbed for 2 h and non-adsorbed virus was discarded. Cells were incubated for 72 h at 37°C under 5% CO_2 after the addition of 0.2 ml Dulbecco's Modified Eagle Medium (DMEM) maintenance medium to each well. The $TCID_{50}$ (50% Tissue Culture Infective Dose) was calculated 3 to 4 days later based on the CPE.

Analysis of the cytotoxicity of chemical constituents

Different concentrations of drugs were added to MDCK or Hela cells in monolayers in 96-well plates in quadruplicate. Cells were subsequently incubated at 37°C under 5% CO_2 for 72 h, and 20 □ l 5 mg/ml methyl thiazolyl tetrazolium (MTT) solution was added to each well. Supernatants were discarded after 4 h and DMSO (0.2 ml) was added, then OD value was calculated using the 570 to 630 nm dual-wavelength method. Cell viability and the median toxic concentrations (TC_{50}) were determined. The maximum non-toxic concentration was used as the initial concentration, and serial ten-fold dilutions were generated for the antiviral assay.

Cell viability = (mean OD value of the drug group / mean OD value of the control group) × 100%.

Antiviral assay

A total of four groups were used: the blank control group, the virus infection group, the ribavirin positive control group and the experimental drug group. MDCK or Hela cells were seeded in 96-well plates at a concentration of 1×10^5 ml^{-1}. When the cells grew into monolayers, they were incubated with 50 □ l 100 $TCID_{50}$ virus solution for 2 h. Unadsorbed virus was washed away with phosphate-buffered saline (PBS). Then different concentrations of drugs in 0.2 ml medium were added to each well in quadruplicate. Cells were subsequently incubated at 37°C under 5% CO_2 for 72 h, and 20 □ l 5 mg/ml MTT solution was added to each well. Supernatants were discarded after 4 h incubation. DMSO (0.2 ml) was added, and the optical density (OD) value was calculated using the 570 to 630 nm dual-wavelength method with a reference wavelength of 630 nm. The viral suppression rate and the drug concentration that caused a 50% reduction in viral cytopathic effect (CPE) (IC50) were calculated.

Viral suppression rate = [(Mean OD value of the experimental group - Mean OD value of the virus infection group) / (Mean OD value of the control cell group - Mean OD value of the virus infection group)] × 100%.

RESULTS

The drugs produced the following main toxic effects on MDCK cells: slowed cellular proliferation, an increased amount of granules, an increased refractive index, morphological changes and the disintegration and collapse of some cells. The MTT assay determined that the TC_{50} values for indirubin, indigotin, 4-3(H)quinazolinone, isovitexin, syringin, succinic acid, sinigrin, neohesperidin, isoliquiritigenin, epigoitrin and buddleoside were 8928, 415, 285, 1093, 1275, 27,859, 48,252, 13,317, 6, 5801 and 742 μg/ml, respectively.

Drugs produced the following main toxic effects on Hela cells: slowed cell proliferation, an increased amount of granules, an increased refractive index, morphological changes and the disintegration and collapse of some cells. The MTT assay determined that the TC_{50} values for indirubin, indigotin, 4-3(H)quinazolinone, isovitexin, syringin, succinic acid, sinigrin, neohesperidin, isoliquiritigenin, epigoitrin and buddleoside were 3, 298, 190, 206, 259, 241, 358, 423, 819 μg/ml, 253 and 204 μg/ml, respectively.

To evaluate the anti-viral efficacy of chemical constituents of *R. isatidis*, we selected the effective concentration of 50 μg/ml, because at this concentration the components had no obvious cytotoxicity. Clear cytopathic effects were observed by microscopy in MDCK cells infected with FM1 for 72 h. The following main phenotypes were observed: cell swelling and rounding, increased gaps between cells, shrinkage or rupture of the cell nucleus and the partial or complete collapse of cells in the most severe cases. The formation of syncytial caused by the fusing of infected cells with neighboring cells was the main CPE observed in Hela cells after infection with RSV for 72 h. The suppression rates of indirubin, indigotin, 4-3(H)quinazolinone, isovitexin, syringin, succinic acid, sinigrin, neohesperidin, isoliquiritigenin, epigoitrin and buddleoside against FM1 infection in MDCK cells were 104.81, 63.53, 49.27, 102.07, 45.40, 60.51, 47.43, 29.20, 24.91, 49.51 and 53.00%, respectively (Table 1). Our results showed that indirubin and isovitexin had the best efficacy against FM1 (Figure 1). Only indirubin, isovitexin, syringin, sinigrin and isoliquiritigenin effectively inhibited RSV infection in Hela cells with suppression rates of 14.47, 12.15, 6.80, 17.89 and 44.56%, respectively (Table 1). Among these constituents, isoliquiritigenin and sinigrin had the greatest efficacy (Figure 2).

DISCUSSION

Herbal medicine has attracted more attention as a source of new antibacterial drugs (Alagesaboopathi, 2011; Kamba and Hassan, 2010; Olajuyigbe and Afolayan, 2011). However, there are few reports on the identify-cation of the effective antiviral components of herbal medicine. Influenza is an acute infectious viral respiratory disease that is currently uncontrollable. Influenza affects a person's normal life and frequently leads to serious complications (De-Mateo et al., 2006). The protective

Table 1. Suppression of FM1 influenza virus and RSV by 11 individual constituents.

Name	Virus	Initial concentration (μg/ml)	Suppression rates by different drug concentration/100%					IC50 (mg/ml)
			1:1	1:2	1:4	1:8	1:16	
Indirubin	FM1	50	104.81**	87.85**	58.87**	**44.22	35.39*	10
	RSV	25	-10.51	-4.02	-14.82	-7.15	-13.34	-
Indigotin	FM1	50	63.53**	23.16**	45.45**	41.15**	8.38**	28
	RSV	100	-4.13	-2.92	5.63	14.47	11.13*	0.1
Quinazolinone	FM1	50	49.27**	40.96**	4.46	10.41*	-3.81	70
	RSV	50	-3.39	-2.57	-3.03	-2.59	-1.08	-
Isovitexin	FM1	50	102.07**	35.86**	48.71**	**18.44	14.80*	14
	RSV	100	-4.74	-2.24	2.34	7.65	12.15*	40
Syringin	FM1	100	45.40**	10.31*	1.91	-2.63	-0.59	69
	RSV	100	-4.31	-2.22	-2.07	6.69	6.80*	0.0024
Succinic acid	FM1	50	20.09	34.92*	22.02*	**60.51	5.92	24
	RSV	50	-14.04	-14.14	-13.66	-13.58	-14.18	-
Sinigrin	FM1	100	**47.43	16.78**	42.14*	7.10	17.92*	74
	RSV	100	-6.53	-4.53	-2.03	**12.36	17.89**	0.304
Neohesperidin	FM1	50	29.20**	24.01**	16.92**	4.79	8.19	193
	RSV	100	-40.22	-38.07	-33.02	-6.04	-15.16	-
Isoliquiritigenin	FM1	8	24.91	-5.89	-15.47	-34.07	-5.74	136
	RSV	5	44.56**	33.18**	29.03**	**28.37	**31.29	10
Epigoitrin	FM1	100	49.51**	9.56	20.89*	*11.54	6.49	111
	RSV	50	-11.02	-10.19	-4.15	-9.26	-8.58	-
Buddleoside	FM1	50	53.00**	21.03**	36.05	*23.02	-4.47	25
	RSV	100	-38.48	-35.04	-26.08	-8.74	-0.94	-
Ribavirin	FM1	100	44.74**	-	-	-	-	-
	RSV	0.0625	77.02**	-	-	-	-	-

The t-test was used for statistical analysis. Groups treated with different concentrations of *R. Isatidis* were compared with the virus infection group. The results were considered statistically significant when * P < 0 .05 or **P < 0.01.

abilities of flu vaccines are limited, because the surface antigens of the influenza virus are constantly mutated. In addition, the pharmaceutical agents that are currently used to treat influenza have relatively severe side effects. For example, the clinically used anti-influenza drugs, zanamivir, amantadine and rimantadine, have toxic side effects that can adversely affect the human nervous system (Luscher-Mattli, 2000). Therefore, the development of effective anti-influenza virus medicines with low toxicity is imperative. Traditional Chinese medicines have few side effects and provide certain advantages in the prevention and treatment of influenza virus. *R. isatidis* (Indigowoad root) is a class of traditional Chinese medicine that is commonly used for fever reduction and detoxification. It has been applied clinically for the treatment of influenza for many years and has proven efficacy in the treatment of viral infections.

We selected eleven constituents from *R. isatidis* in three major categories, phenylpropanoid, lignans and organic acids, based on the literature and screened for their antiviral activity. Our results showed that the anti-FM1 potency of the eleven individual *R. isatidis* constituents

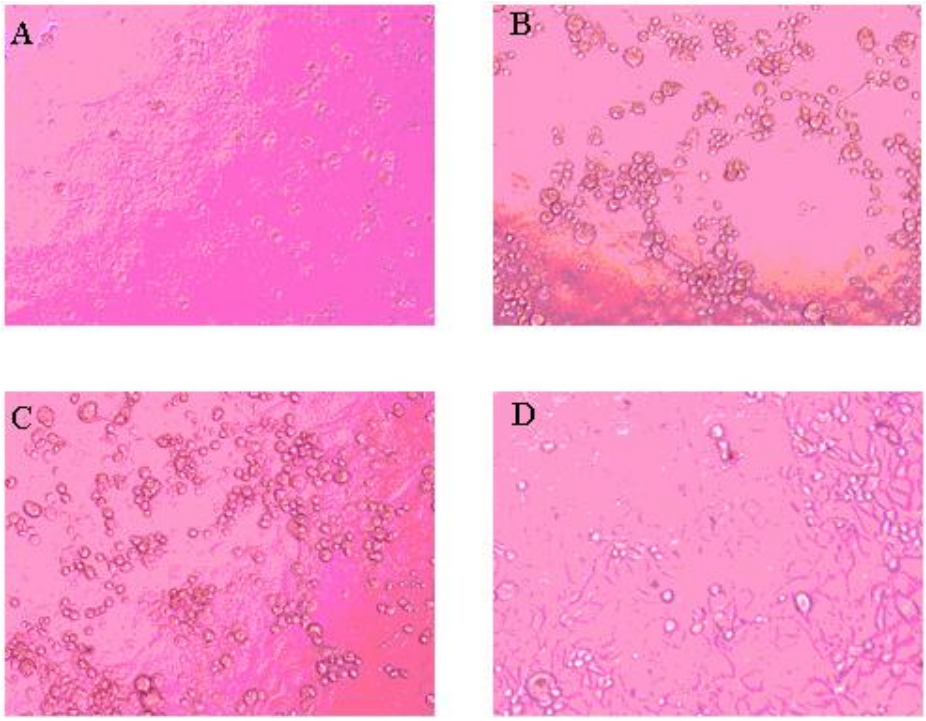

Figure 1. Antiviral activity against FM1 in MDCK cells. A: Normal MDCK cells; B: FMI infected MDCK cells; C: Suppression of FM1 by isovitexin (50 μg/ml); D: Suppression of FM1 by indirubin (50 μg/ml). Bar = 100 μm.

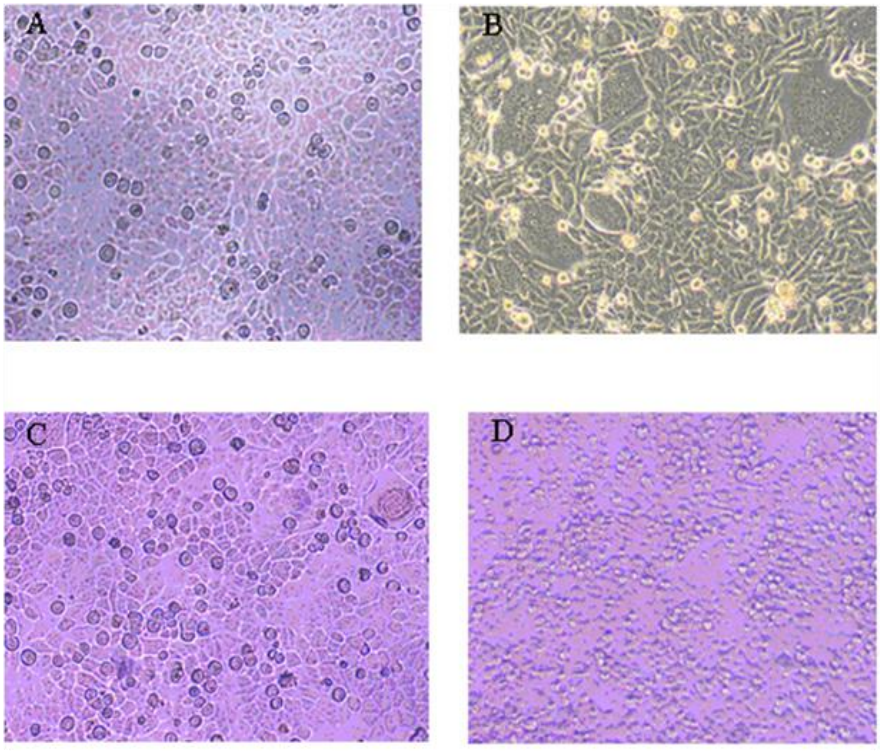

Figure 2. Antiviral activity against RSV in Hela cells. A: Normal Hela cells; B: RSV infected Hela cells; C: Suppression of RSV by sinigrin (6.25 μg/ml); D: Suppression of RSV by isoliquiritigenin (5 μg/ml). Bar = 100 μm.

was indirubin > isovitexin > indigotin > succinic acid > buddleoside > epigoitrin > 4-3(H)quinazolinone > sinigrin > syringin > neohesperidin > isoliquiritigenin. Among these constituents, indirubin and isovitexin had the strongest anti-FM1 effects, which were better than the positive control drug. Isovitexin, buddleoside, syringin, neohesperidin and isoliquiritigenin are in the phenylpropanoid and flavonoids families. No previous studies have screened these components of *R. isatidis* for antiviral activity.

The anti-RSV potency of 5 individual *R. isatidis* constituents was isoliquiritigenin > sinigrin > indigotin > isovitexin > syringin. Indirubin, succinic acid, buddleoside, neohesperidin, 4-3(H)quinazolinone and epigoitrin had no anti-RSV effect.

Conclusion

Based on the experimental results, we conclude that the eleven constituents had stronger antiviral effects against FMI than RSV. Domestic and foreign scholars have performed a large number of studies on the anti-viral function of *R. isatidis*. However, the identity of the active ingredient of *R. isatidis* that plays a leading role in virus inhibition virus and the anti-viral mechanism of *R. isatidis* remains unclear. In-depth research is required in this field. Basic research on the chemical constituents of *R. isatidis* that are responsible for fever reduction and detoxification is lagging, which severely restricts the application and development of *R. isatidis*-based medicine. Therefore, a systematic study and thorough elucidation of the active anti-viral ingredients of *R. isatidis* and an exploration of the underlying mechanism can produce huge social and economic benefits with important theoretical and practical significance in the development of new anti-viral drugs, the rationalization of the preparation process of medicine, the scientific optimization of quality control methods and the rational use of *R. isatidis* resources.

ACKNOWLEDGEMENTS

This study was supported by Jiangsu province joint innovative research funding programs (BY2009109); Jiangsu college advantages of discipline construction project funded projects; Jiangsu province college graduate research and innovation projects; the National Natural Science Foundation (81073023); National Ministry of Education of new teachers Fund (20093237120015).

REFERENCES

Alagesaboopathi C (2011). Phytochemical analysis and antimicrobial evaluation of Andrographis lineata Nees leaves and stem extracts. Afr.J.Pharm.Pharma.,5:http://www.academicjournals.org/AJPP/abstracts/abstracts/abstract 2011/February/Cwala et al.htm. pp.1190-1195.

De-Mateo S, Larrauri A, Mesonero C (2006). Influenza Surveillance. New Solutions to an old problem. Gal. Sanit., 20: 67-73.

Kamba AS, Hassan LG (2010). Phytochemical screening and antimicrobial activities of *Euphorbia balsamifera* leaves, stems and root against some pathogenic microorganisms. Afr. J. Pharm. Pharm., 4: 645–652.

Kong W, Zhao Y, Shan L, Xiao X, Guo W (2008). Thermochemical studies on the quantity-antibacterial effect relationship of four organic acids from Radix Isatidis on Escherichia coli growth. Biological Pharmaceut. Bull., 31: 1301-1305.

Luscher-Mattli M (2000). Influenza chemotherapy: A review of the present state of art and of new drugs in development. Arch. Virol 145: 2233- 2248.

Olajuyigbe OO, Afolayan AJ (2011). *In vitro* antibacterial activities of crude aqueous and ethanolic extracts of the stem bark of Acacia mearnsii the Wild. Afr. J. Pharm. Pharm., 5: 1234-1240

Qing C (2009). Pharmacological effects and clinical application of Radix Isatidis. Chinese Pharmaceutical Affairs. 23: 607-608.

Shin EK, Kim DH, Lim H, Shin HK et al (2010). The anti-inflammatory effects of a methanolic extract from Radix Isatidis in murine macrophages and mice. Inflammation. 33: 110-118.