Plant Science, 75 (1991) 161—165
Elsevier Scientific Publishers Ireland Ltd.

Occurrence of some antiviral sterols in *Artemisia annua*

M.M. Abid Ali Khan*, D.C. Jain, R.S. Bhakuni, Mohd. Zaim and R.S. Thakur

Central Institute of Medicinal and Aromatic Plants (CIMAP-CSIR), P.O. RSM Nagar. Lucknow-226016 (India)

(Received December 29th, 1989; revision received April 18th, 1990; accepted January 2nd, 1991)

Out of the twenty one medicinal plants evaluated for their virus inhibitory activity against tobamoviruses on their test hosts reacting hypersensitively, extracts of *Lawsonia alba, Artemisia annua* and *Cornus capitata* showed high virus inhibitory activity. The virus inhibitory agent (s) occurring in *A. annus* plant was isolated by conventional methods and identified as sterols. The sterols were characterized by spectral methods as sitosterol and stigmaterol.

Key words: tobamoviruses; *Artemisia annua;* virus inhibitory agent(s); β-sitosterol; stigmasterol

Introduction

Recent investigations on inhibition of plant viruses have shown that strong virus inhibitors occur in a limited number of plants [1—7]. However not much effort has been made to purify and characterize the active agents from these phytoextracts. There are still fewer reports which deal critically with their isolation and characterization [2,8—10]. Therefore, in the present investigation, some medicinal plants were initially evaluated for their antiviral activity. Then the virus inhibitory agents(s) (VIA) present in *Artemisia annua* was isolated and identified.

Material and Methods

Virus cultures and test plants

The cultures of tobamoviruses viz. tobacco mosaic virus and sunnhemp rosette virus were maintained on their hosts reacting systematically viz. *Nicotiana tabacum* var. Np 31 and *Crotalaria juncea,* respectively, and test plants viz. *Datura stramonium* L. and *Cyamopsis tetragonoloba* Taub. were raised in compost soil in a glasshouse.

Virus inoculum and inoculation

The young diseased leaves of tobacco or sunnhemp plant were ground with mortar and pestle with distilled water used as diluent. The pulp obtained was squeezed through muslin cloth and centrifuged at 3000 rev./min for 10 min. The supernatant was diluted to 1:100 (w/v) and used as inoculum.

Inoculation was done with a forefinger using carborundum powder (600 mesh) as an abrassive.

Extraction of VIA

The fresh plant material (10 g) was ground in mortars with 20 ml of distilled water. The pulp obtained was squeezed through muslin cloth and centrifuged at 300 rev./min for 10 min, made up to the dilution 10^{-1} w/v by adding distilled water and used for experiments. Similarly, the 10 g of fresh plant material marked with (a) in Table I were ground in 20 ml of ethanol while the plants marked with (b) in Table I were ground with a mixture of ethyl acetate/hexane (70:30). The pulp obtained was squeezed through muslin cloth and centrifuged at 3000 rev./min for 10 min, made up to the final dilution to 10^{-1} w/v and used for experiments (Table I).

Antiviral evaluation

The virus inhibitory activity was evaluated by the application of test samples on 3 lower leaves of 10 healthy *D. stramonium* or *C. tetragonoloba* test plants. Similarly 3 leaves of 10 control plants were

Correspondence to: Dr. M.M. Abid Ali Khan, Department of Botany, Lucknow University, Lucknow-226007. (U.P.), India.

0168-9452/91/$03.50

Table I. Evaluation of some medicinal plants for their virus inhibitory activity against tobamoviruses. Inhibitors of plants marked with (a) were suspended in ethanol and plants marked with (b) were suspended in a mixture of ethyl acetate and hexane and assayed against SRV on *C. tetragonoloba* (X) and TMV on *D. stramonium* (Y) plants.

Sl. no.	Plant extract	Family	Part screened	Percent virus inhibitory activity
1	*Apium graveolens* L.	Apiaceae	Leaf	59 (Y)
2	*Artemisia annua* L. (b)	Asteraceae	Whole plant	73 (Y)
3	*Bixa orellana* L. ()	Bixaceae	Leaf	24 (X)
4	*Clerodendrum phlomidis* L.f. (a)	Verbenaceae	Leaf	29 (X)
5	*Costus speciosus* (Koen.) Sm. (a)	Zingiberaceae	Leaf	54 (X)
6	*Cornus capitata* L.	Coilnaceae	Leaf	78 (Y)
7	*Digitalis latana* Ehrh.	Scrophulariaceae	Leaf	Nil (Y)
8	*Gardenia gummifera* L.f.	Rubiaceae	Leaf	62 (Y)
9	*Jataropha guricus* L.	Euphorbiaceae	Leaf	40 (Y)
10	*Kalanchee spathulata* DC.	Crassulaceae	Leaf	49 (Y)
11	*Lepidium sativum* L.	Cruciferae	Whole plant	34 (Y)
12	*Lawsonia alba* Lam. (a)	Lythraceae	Seeds	97 (Y)
13	*Melochia corchorifolia* L.	Sterculiaceae	Leaf	Nil (Y)
14	*Mentha spicata* L. (a)	Labiatae	Whole plant	10 (X)
15	*Pluchea lanceolata* Oliver & Hiern. (a)	Asteraceae	Leaf	33 (X)
16	*Phyllanthus neruri* Auct. (a)	Euphorbiaceae	Whole plant	07 (X)
17	*Rauvolfia canescens* L.	Solanaceae	Fruit	30 (Y)
18	*Sedum* sp. Wall.	Grassulaceae	Leaf	21 (Y)
19	*Silybum marinum* L. Gaertn.	Asteraceae	Leaf	Nil (Y)
20	*Spilanthes acemella* L.	Asteraceae	Leaf	28 (Y)
21	*Vitex negundo* L. (a)	Verbenaceae	Leaf	29 (Y)

rubbed with distilled water, ethanol or ethylacetate and hexane mixture which served as control sets. After 24 h of treatment, all the leaves of test plants were washed with distilled water, dusted with carborundum powder (600 mesh) and inoculated with TMV or SRV as required for their test host. The local lesions were counted 3—5 days after virus challenge.

Calculations

The percent of virus inhibitory activity was calculated by using the formula IP = $(1 - A/B) \times 100$, where IP = inhibition percentage; A = average number of local lesions on treated leaves; B = average number of lcoal lesions on control leaves. The calculation of significance of data were same as described earlier [11].

Isolation and partial characterization of IVA from A. annua

This was carried out by using conventional methodology described earlier [12—16].

Physicochemical properties. The properties were studied on *C. tetragonoloba* against sunnhemp rosette virus.

Storage. β-Sitosterol and stigmasterol (1 mg/ml) were stored at laboratory temperature (36 ± 5°C) in stoppered sterilised test tubes and VIA of the samples was tested regularly at intervals of 10, 20, 30 and 40 days.

Dilution. β-Sitosterol and stigmasterol were diluted to 5×10^2, 1×10, 10^2 and 0.1 μg/ml separately and VIA of diluted samples was tested.

Spectra were recorded with the instruments of IR Perkin Elmer-399 B model ^{1}H-NMR and ^{13}C-NMR of varian FT-80A in $CDCl_3$ with TMS as an internal standard. GC-Mass system 1020 B of Finnigan MAT.

Results

Initially extracts from some medicinal plants were screened for their virus inhibitory activity against the tobamoviruses, tobacco mosaic and

sunnhemp rosette viruses on their respective test hosts reacting hypersensitively. This preliminary screening revealed that aqueous extracts of *Cornus capitata* (Collnaceae), *Apium graveolens* (Apiaceae), *Gardenia gummifera* (Rubiaceae), alcoholic extract of *Lawsonia alba* (Lythraceae), *Costus speciosus* (Zingiberaceae) and a hexane-ethylacetate extract of *A. annua* reduced virus infection to variuos degrees in their respective local lesion hosts when applied 24 h prior to virus challenge. Extracts from *L. alba, C. capitata* and *A. annua* plant afforded the maximum reduction in tobamoviruses infection while the extracts of *A. graveolens, G. gummifera* and *C. speciosus* were somewhat less active. The rest of the plants had no significant antiviral response (Table I).

The VIAs present in *A. annua* plant were isolated by conventional methodology.

Dried and powdered whole plant of *A. annua* (2 kg) were extracted with *n*-hexane (6 × 2.5 l) at room temperature. The hexane extract obtained was concentrated under reduced pressue and after removal of the solvent yielded 54 g of this material 50 g was applied to a silica gel column (105 × 7.5 cm) (500 g) and eluted in a step-wise fashion with *n*-hexane-ethylacetate (95:5, 90:10, 85:15 and 80:20). Fifty fractions of 500 ml each were collected and monitored by TLC. Fractions giving similar chromatogram were pooled. These fractions were further purified by repeated chromatography on 105 × 7.5 cm column yielded 9 compounds which were identified as tetra triaconitane, nonacosanol, octacosanol, artermisinin, deoxyartemisinin, arteannuan-B, hentriacontanyl triacontanoate, 2-methyl tricosan-8-one-23-ol and sterols by comparison with spectral data reported in the literature. The above method yielded 9 crystalline compounds which were identified and tested for their antiviral activity (Table II). Sample no. 5 was identified as a sterol and exhibited high virus inhibitory activity. Other identified compounds were comparatively very less active.

The sterol mixture (Fraction no. 5) was separated and identified as follows.

Characterization of active principle

The active fraction was obtained as a crystalline mixture during the column chromatography of hexane extract of *A. annua.* The homogenity of these compounds was ascertained by repeated crystallization which resulted in material showing well defined spot on TLC plates but which did not show sharp melting points. The mass spectra of the mixture showewd two molecular ion peaks at *m/c* 414 and 412. The ^{13}C-NMR of the compound revealed 36 signals of which 29 are identical to those of β-sitosterol. The remaining 7 signals can be assigned to C-22 to C-25 and C-28 of stigmasterol by comparison with the reported values [16]. The chemical shifts of olifinic methene resonances appearing at 138.51 and 129.53 are of particular value in differentiating these compounds.

The sterols were further identified by gas chromatography (GC) using 6 ft. × 1/8 in. stainless steel (SS) column packed with 3% silicon OV-17

Table II. Virus inhibitory activity of some compounds purified from *A. annua.* Data significant (a) at 1% level and (b) at 5% level.

Sl. no.	Name of compounds	M.P. (°C)	Mol. wts.	% inhibitory activity
1	Tetratriaconitane	68	478	40 (b)
2	Nonacosanol	75—76	424	48 (b)
3	Octacosanol	73—74	410	46 (b)
4	Hentriacontanyltriacontanoate	82—83	886	43 (b)
5	Sterols mixture	136—147	412—414	72 (a)
6	2-Methyltricosan-8-one-23-Ol	86—87	368	50 (a)
7	Artemisinin	153—154	282	40 (b)
8	Deoxyartemisinin	114—115	266	56 (a)
9	Arteannuan-β	150—151	248	40 (b)

Table III. Virus inhibitory activity of β-sitosterol and stigmasterol purified from *A. annua*. Data significant (a) at 1% level.

Name of steroidal compound	M.P. (°C)	Mol. wt.	% inhibitory activity
β-Sitosterol	136	414	80 (a)
Stigmasterol	167	412	64 (a)

(Methyl phenyl silicon for gas chromatography) on chromosorb W HP, (supplied by All tech. Associate Inc. U.S.A.) temperature programmed at isothermal 250°C for 2 min then 4°C per min to 290°C. Reference samples of β-sitosterol and stigmasterol exhibited the same retention time.

The sterol fraction was acetylated with acetic anhydride in pyridine in the usual way. The acetate mixture was analysed by TLC on silica gel plates (containing 20% silver nitrate with benzene/hexane (4:6). The plate was run 3 times and sprayed with anisaldehyde and visualized by heating. Hydrolysis of the acetate yielded the free sterol, m.p. 136°C, of identical IR, NMR and mass, as β-sitosterol and another sterol, m.p. 167°C, identified in IR, NMR and mass to stigmasterol. The purified β-sitosterol fraction showed 80% virus inhibitory activity while the stigmasterol showed 64% activity (Table III).

Experiments for physical characteristics are summarized in Table IV which reveal that the inhibitory activity of these sterols was gradually lost when stored in vitro at room temperature (32 ± 5°C) in closed vials. The sterols were active up to a dilution of 100 μg/ml, the activity was completely lost on diluting the samples by 10 μg/ml. The results clearly indicate that long storage and increasing dilution of these sterols gradually decreased the antiviral activity (Table IV).

Discussion

Antiviral screening of higher plants has shown that some of them contain highly potent inhibitors of plant viruses which display varying degree of inhibition [3—7,17—20]. This during our screening programme *A. annua, L. alba* and *C. capitata* plants were found to possess virus inhibitors.

Only a very few inhibitors have been fully characterized, and these show striking chemical variation. Some inhibitors were characterized as glycoproteins [9], protein [2,21] and polypeptide [8] in nature while other inhibitors showed the characteristics of carbohydrates or polysaccharides [10]. Similarly the virus inhibition by strawberry and raspberry leaf extract was due to the presence of phenolic tannins and the inhibitor of *Begonia tuberhybrida* was identified as oxalic acid. In stone fruit plants e.g. *Prunus* sp. the inhibitor were of flavonoid in nature and related to guercitin [3,20,22]. Similarly the steroidal, triterpenoid glycosides and volatile constitutents of plant origin have been shown to possess virus inhibitory activity [23—25]. The range of their molecular weights varied greatly. However, the nature of a virus inhibitor present in *A. annua* was identified as a mixture of low molecular weight sterols which was further separated and identified as sitosterol and stigmasterol. Further the suggestion that these antiviral sterols affects hosts rather than viruses is supported by the fact that the

Table IV. Effect of storage and dilution on virus inhibitory activity of β-sitosterol and stigmasterol. Data significant (a) at 1% level and (b) at 5% level.

Name of sterols	% Inhibitory activity							
	Storage in days				Dilutions (μg/ml)			
	10	20	30	40	500	100	10	11
β-Sitosterol	71 (a)	67 (a)	60 (a)	58 (b)	73 (a)	40 (b)	Nil	Nil
Stigmasterol	55 (a)	48 (b)	42 (b)	37 (b)	58 (a)	39 (b)	Nil	Nil

number of lesions produced are reduced when the compound applied first followed by virus inoculation and evidence was found for reduction of lesion size viz. diameter of lesion. This is the first report on sterols present in *A. annua* plant showing virus inhibitory activity.

References

1 H.N. Verma and M.M. Abid Ali Khan, Management of Plant virus diseases by *Pseuderan themum bicolor* leaf extract. *Zeitschrift* fur *Pflanzenkrankheiten* und *Pflanzenschutz.* 9 (3) (1984) 266—272.

2 H.N. Verma and M.M. Abid Ali Khan, A soluble protein from non host plant stimulating systemic resistance in host. Nat. Symp. Role of Biotech. Crop Protect. Kalyani (India) (1987) 23—24.

3 H.N. Verma, Interaction like antivirual agents from plants, in: A. Verma and J.P. Verma (Eds.), Vistas in Plant Biology, Malhotra Publishing House, New Delhi, 1986, pp. 481—490.

4 A. Hiramatsu, K. Norifumi and O. Naofumi, Properties of two inhibitors of plant virus infection from fruiting bodies of Lentinus edodes and from leaves of *Yucca recurvifolia* salisb. Agric. Biol. Chem., 51(3) (1987) 897—904.

5 F. Kalo and T. Taniguchi, Properties of a virus inhibitor from spinach leaves and mode of action. Ann. Phytopathol. Soc. Jpn., 53(2) (1987) 159—167.

6 W.D. Ostermann, U. Meyer and R.M. Leiser, Induction of plant virus resistance. 2. Leaf extract from carnation plants (*Dianthus caryophyllus* L.) as inducer of resistance. *Zentralbl Mikrobiol.,* 142(3) (1987) 229—238.

7 Z.B. Zaidi, V.P. Gupta, A. Samad and Q.A. Naqvi, Inhibition of spinach Mosaic virus by extract of some medicinal plants. Curr. Sci., 57(3) (1988) 151—152.

8 R.A. Owens, G. Bruening and R.J. Shepherd, Virology, 56 (1973) 390—393.

9 H.N. Verma and L.P. Awasthi, Antiviral activity of *Boerhavia diffusa* root extract and the physical properties of the inhibitor. Can. J. Bot., 57 (1979) 926—932.

10 G. Worms and F. Nienhaus, Characterization and effect of virus inhibiting polysaccharide from cabbage plants. Phytopathol. Z., 82 (1975) 224—225.

11 G.W. Snedecor, Statistical Methods. Allied Pacific (P) Ltd., Bombay, India, 1961.

12 A. Ulubelen and B. Helfen, Phytochemcial investigation of the herb of *Artemisia annua.* Planta Med., 29(3) (1976).

13 D. Jerenic, A. Jokie, A. Behbud and M. Stefanovie, New type of sesquiterpene lactone isolated from *Artemisia annua* Tetrahedron letters. 32 (1973) 3039—3042.

14 You-You. Tu, Qieong Zhu and Xing Shen, Constituents of young *Artemisia annua.* Zhongyao Tongbao 16(9) (1985) 419—420.

15 Jing-Ming, Liu, Nimu-Yun, Ju-fen. Fan, You-You. Tu, Wu-Zhao-Hua, Yu-Lin. Wu, Wei-Shan.Chou, Structure and reaction of arteannum-B, Hua Xue Xuebao, 37(2) (1979) 129—143.

16 J.L.C. Wright, A.G. McImes, S. Shimizu, D.G. Smith, J.A. Walter, D.R. Idler and W. Khalil, Identification of C-24 alkyl epimers of marine sterols by C-13 Nuclear Magnetic resonace spectroscopy. Can. J. Chem., 56 (1979) 1898—1903.

17 H.N. Verma, M.M. Abid Ali Khan and S.D. Dwivedi, Biophysical properties of highly antiviral agents present in *Pseuderanthemum atropurpureum* and *Bougainvillea spectabilis* extracts. Ind. Plant Pathol., 3(1) (1985) 13—20.

18 N.S. Murty and K. Nagarajan, Role of Plant extracts in the control of TMV infection in nursery and field grown tobacco. Ind. Phytopathol., 39(1) (1986) 98—100.

19 B.P. Pandey and J. Monan, Inhibition of turnip mosaic virus by plant extracts. Ind. Phytopathol., 39(3) (1986) 489—491.

20 A.D. Bobyr, A.A. Barkalova and L.T. Zhmurko, Effect of certain biologically active substances on beat mosaic virus and bean yellow mosaic virus. Mikrobiol. Z.H. (Kiev), 49(5) (1987) 63—66.

21 O. Naofumi and A. Hiramatsu, Purification and chemical properties of an inhibitor of plant virus infection from leaves of *Yucca recurvifolia* salisb. Agric. Biol. Chem., 51(3) (1987) 841—896.

22 H.N. Verma, L.P. Awasthi and K. Mukerjee, Characteristics and mode of action of inhibitors of virus infection, in: K.S. Bilgrami, P.S. Misra and R.S. Misra (Eds.), Advancing Frontiers of Mycology and Plant Pathology, Today & Tomorrow's Printers and Publishers, New Delhi, India, 1982, pp. 151—159.

23 H.S. Shukla, G.P. Rao and S.C. Tripathi, Reduction in potato virus-X and potato virus-Y infectivity by volatile constituents. Anal. Edafol. Agrobiol., 44 (1985) 1763—1766.

24 I.N. Choban, A.S. Dimoylo, I.B. Bersuker, I.T. Balashova and P.K. Kintya, Structure-activity correlations for antiviral properties of steroidal glycosides. F.E.C.S. Third International Conference on Chemistry and Biotechnology of Biologically active natural products. September 16—21, Bulgaria (Sofia), 5 (1985) 431—435.

25 D.C. Jain, M.M. Abid Ali Khan, Mohd. Zaim and R.S. Thakur, Antiviral evaluations of some steroids and their glycosides of medicinal plant origin: A new report. Natl. Acad. Sci. Lett., 13(2) (1990) 41—42.