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EFFECTS OF HERBAL PREPARATIONS ON THE PROPAGATION OF INFLUENZA VIRUSES

I. EFFECTS OF HERBAL PREPARATIONS ON THE PROPAGATION OF INFLUENZA VIRUSES IN CULTURES OF CHICKEN EMBRYO FIBROBLASTS AND IN CHICKEN EMBRYOS

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26 herbal preparations were examined whereof 12 suppressed the flu virus propagation in cell cultures or in chicken embryos.

In recent years many observations have been made concerning the antiviral activity of herbal preparations.

Suppressive effects of plant drugs on the propagation of flu virus have been demonstrated for herbal drugs classified as expectorants, anti-inflammatory agents, diaphoretics, coating agents (1-3) and tannic agents (4-10).

There are numerous reports concerning antiviral activity of herbal preparations and suppression of virus propagation *in vivo*.

The Honolulu researchers (11, 12) have focused their efforts on plants commonly used in folk medicine some of which suppressed propagation of viruses. Suppressive effects have also been demonstrated for extracts from leaves of blue gum tree (13-16), common bahu and peppermint (17-20).

Leaf extracts of higher plants were studied by *Banchero et al.* (21-24).

The results of such observations gave an incentive to investigate the effects of herbal preparations widely used for therapeutic purposes on propagation of type A_2 and type B flu viruses.

EXPERIMENTAL PART

Materials and methods

V i r u s e s . Influenza viruses A_2 (England 64) and B (Johannesburg 58) were obtained from Department of Virology, State Hygiene Institute in Warsaw. Viruses were propagated in chicken embryos, titrated in fibroblast culture and stored in vials at –20 °C. The titre value $TCID_{50}$ (tissue culture 50% infectious dose) was calculated using the Reed and Muench formula (25).

H e r b a l p r e p a r a t i o n s . Decoctions of the following therapeutic herbs purchased in the herbal stores of the market chain "Herbapol", Poland were investigated:

1. *Anthodium Chamomillae* — capitulum of common chamomile; 2. *Flos Calandulae* — flower of pot marigold; 3. *Hb. Herniariae* — herb of rupture wort; 4. *Hb. Hyperici* — herb of St. John´s wort; 5. *Fol. Mellisae* — leaf of common bahu; 6. *Fol. Menthae piperitae* — leaf of peppermint; 7. *Flos Millefolii* — flower of yarrow; 8. *Hb. Origani* — herb of marjoram; Pektosan — herbal mixture; 10. Pyrosan — herbal mixture; 11. *Flos Primulae* — flower of primrose; 12. *Flos Salviae* — leaf of sage; 13. *Flos Sambuci* — flower of elder; 14. *Fruct. Sambuci* — fruit of elder; 15. *Rdx. Saponariae* — root of soapwort; 16. Septosan — herbal mixture; 17. *Hb. Serpylli* — herb of wild thyme; 18. *Rdx. Symphyti* — root of common comfrey; 19. *Inflorescenti Tilliae* — inflorescence of linden; 20. *Hb. Thymi* — herb of common thyme; 21. *Flos Verbasci* — flower of common mullein; 22. *Hb. Violae tricoloris* — herb of wild pansy.

Juice of:

23. *Allium sativum* — tuber of garlic; 24. *Fructus Oxycocci* — fruit of cranberry; 25. *Fructus Viburni opuli* — fruit of guelder rose; 26. *Fructus Vitis idae* — fruit of cowberry. The decoctions were prepared according to Polish Pharmacopoeia FP IV. They were sterilized by filtering through the membrane filters Coli 5 and stored in the refrigerator at 4 °C up to 7-10 days.

To obtain the juice, the thoroughly cleaned fruits of guelder rose, cowberry and cranberry were squashed/crushed in a mortar. The juice produced was pressed through the gauze and eventually filtered through the membrane filters. The juices were stored at 4 °C up to 2-3 weeks.

Storage conditions for garlic juice

Garlic bulbs were stripped off the outer theca and then squashed in a sterilized mortar. The juice obtained was pressed through a sterilized gauze into a sterilized pot. The juice was incubated for 1 hour at 37 °C to convert non-active alliin into active allicin, and eventually stored at –10°C. The juice stored at these conditions retains antiviral activity during 6 months.

Cultures of chicken embryo fibroblasts

Trypsinized cells (c. 300,000 cells/cm^3) of chicken embryos were cultured on RTN medium (Hanks solution + 0.5 % lactalbumin hydrolysate) with addition of 2% calf serum. Once the cell monolayer was obtained, the culture was rinsed with PBS solution and overlaid with Earle medium. Replication of viruses in the cell culture was visualized by means of the hemadsorption reaction (HAS) acc. to *Vogel* and *Schelokov* (26).

Toxicity assessment. The decoctions and juices in study were diluted with physiologic salt solution to obtain a series of aliquots (geometrical progression) which were then added to the cell cultures. 3 test tubes of tissue culture were used for each concentration of preparation. The cultures were incubated over 4 days at 37 °C. The pH value of the decoctions in study was c. 7. The pH value of the juices was adjusted to 7 with NaOH solution.

Degenerative changes appearing in cells of the tissue culture as compared with a tissue not exposed to the preparations were ascertained as a manifestation of the toxic activity of the preparation.

Determination of effects of herbal preparations on the full development cycle of type A_2 and B influenza viruses in the culture of chicken embryo fibroblasts

An amount of 0.1 cm^3 of each preparation in its maximum non-toxic concentration in respect to the cultured cells was added to the 48 hour old culture of chicken embryo fibroblasts. 6 test tubes with a cell culture were used for each preparation: 3 infected with type A_2 flu virus and a further 3 with type B flu virus. The virus dose was 100 $TCID_{50}$. 2 series of 3 test tubes containing cell culture infected with type A_2 or B flu viruses respectively with no addition of preparations were used as a virus reference. Non-infected cultures were used as a tissue reference. After 48 hours of incubation at 37 °C a hemadsorption reaction test was made.

Determination of effects of herbal decoctions on the adsorption stage of viruses on the cells of the tissue culture

Various concentrations of viruses were added together with the preparations in study into the cultures of chicken embryo fibroblasts. After a 30 minute incubation, sufficient to absorb the viruses into the cells, the liquid was removed from the culture to discontinue the contact between the virus and the preparation. Eventually the cultures were rinsed with the buffered physiologic salt solution to remove the remaining amounts of the preparations and the infected cell cultures were placed in an incubator. After 48 hours of incubation a HAS reaction test was carried out and the infectious dose (titer) of viruses was calculated ($TCID_{50}$).

Determination of effects of herbal preparations on the intracellular propagation of viruses

The virus infected cultures were incubated for 1 hour at a room temperature to induce the adsorption and penetration of viruses into the cells. Next, the preparations were added and after the incubation of 2 hours at 35 °C the solution was removed from the test tubes to discontinue the activity of the preparations. After the cell cultures were rinsed a support medium was added and the cultures were left in the incubator for a further 48 hours. In this way the effects of preparations on the initial stages of the intracellular propagation of viruses were determined.

To investigate the influence of the decoctions and juices on the latter stages of virus propagation the infected cultures were incubated at 35 °C for 2 hours and preparations were added thereafter. The cultures prepared in this way were left in an incubator and the virus titer was eventually calculated.

Investigation of preventive effects of preparations in cell cultures

The preparations were added to the cultures of chicken embryo fibroblasts for the duration of 2 hours. Then the liquid was removed from the culture, the culture was rinsed three times with PBS solution and infected with

viruses. After a 48 hour incubation time-period a hemadsorption reaction test was carried out and virus titer was calculated.

Investigation of virucidal effects

To demonstrate the direct virucidal activity of preparations, the decoctions were mixed up in equal quantities with the virus and incubated for 2 hours at 37 °C. Afterwards the samples were diluted to the concentration where the preparation had no virucidal effect and the virus dose was ca. 100 $TCID_{50}$. The material prepared in this way was used to infect tissue cultures and virus titer was eventually calculated.

Investigation of preventive effects of decoctions on the propagation of influenza viruses in chicken embryos

0.2 cm^3 volumes of each herbal preparation in non-toxic concentrations were injected into the allantoic cavity of 10-12-day chicken embryos. After a 2 hour incubation at 37 °C the embryos were infected intraallantoically with type A_2 and B viruses in doses of 100 $TCID_{50}$. The incubation was carried out over 48 hours at 35 °C. Thereafter the embryos were placed for 12 hours in a refrigerator at 4 °C.

Finally, the virus titers for the cultures of chicken embryo fibroblasts were determined in the collected liquids and the hemagglutination assay tests were made. For the hemagglutination reaction tests according to Takatsa, organic glass plates were used. The 0.5 % suspension of chicken blood cells was added to the series of aliquots (double dilution) prepared from the liquid collected and after an incubation time of 30 minutes the results were determined. The hemagglutinantion titer was calculated as a mean titer value for 3 embryos.

Investigation of effects of herbal preparations on the propagation of flu viruses in chicken embryos

(Effects on the full development cycle of a virus)

0.2 cm^3 volumes of virus at the dose of 100 $TCID_{50}$ and 0.2 cm^3 of preparation at the maximum non-toxic concentration for the chicken embryos were injected simultaneously into the allantoic cavity of chicken embryos. The preparations were acting during the full development cycle of the virus. After 48 hours of incubation both the virus titer and the hemagglutination titer were determined in the collected allantoic fluid.

Following this experiment the effects of the preparations on the intracellular propagation of influenza virus were investigated. For this purpose the preparations were added 2 hours after infecting the embryos with the virus. After a further 48 hours the fluid was collected from the embryos and both the hemagglutinantion titer and the virus titer were determined.

Investigation of virucidal effects

To demonstrate the direct effects on the viruses, the herbal preparations were mixed in equal quantity with the flu virus and preincubated for 2 hours at 37 °C. After 2 hours the sample was diluted to obtain the concentration of the decoction below the suppression threshold for the propagation of viruses, with a virus concentration of ca. 100 $TCID_{50}$. The embryos were then infected intraallantoically by injection of 0.2 cm^3 virus suspension preincubated with the decoction. After an incubation period of 48 hours the allantoic fluid was collected and both the infectious titer for the chicken embryo fibroblast culture and the hemagglutination titer of the virus were determined.

RESULTS OF THE STUDY

Of 26 preparations added to the infected cells for the duration of the full developmental cycle of the virus, 12 preparations suppressed the development of the type A_2 flu virus, reducing the virus titer by 4.9 up to 1.9 log compared with the virus reference.

These were the decoctions of the following herbal materials: flower of common mullein, Septosan, garlic juice, herb of wild thyme, flower of elder, herb of common thyme, root of soapwort, flower of yarrow, herb of St. John´s wort, flower of primrose, root of common comfrey and herb of rupture wort. The results are illustrated in the Fig. 1.

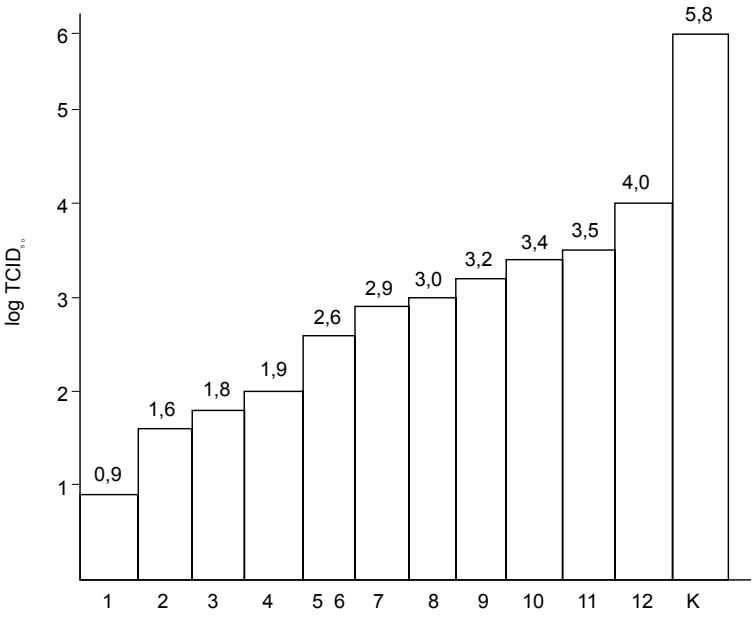

Fig. 1. Effects of herbal preparations on the development of A_2 type flu viruses in the cultures of chicken embryo fibroblasts (effects on the full developmental cycle of the virus).
1 – Dec. Flos Verbasci
2 – Dec. Septosani
3 – Succus Allium sativum
4 – Dec. Hb. Serpylli
5 – Dec. Flos Sambuci
6 – Dec Hb. Thymi
7 – Dec Rdx. Saponariae
8 – Dec. Flos Millefolii
9 – Dec. Hb. Hyperici
10 – Dec. Flos Primuli
11 – Dec. Rdx. Symphyti
12 – Dec. Hb. Herniariae
K - reference

According to Fig. 1 the strongest suppressive activity was revealed by the decoction of flower of common mullein, which at a concentration of 1% reduced the titer of A_2 type flu virus by 4.9 log, whereas the weakest effect was observed for the decoction of herb of rupture wort, which reduced the virus titer by 1.8 log.

Suppressive effects on B type flu virus were observed for the decoctions of flower of common mullein, flower of elder, flower of yarrow and garlic juice.

In the further experiments attempts were made to determine the minimal active doses of the preparations by reducing their concentrations in cultures from 1% to 0.1%. It was demonstrated that all the preparations showed antiviral activity only in their maximum non-toxic concentrations.

Further tests were designed to determine the stage of the viral infection of cells at which the preparations develop their activity. Of 12 preparations showing suppressive effects on the full development cycle of viruses 6 decoctions made of herb of wild thyme, flower of common mullein, root of common comfrey, herb of St. John´s wort, flower of primrose and herb of rupture wort suppressed the absorption of A_2 type flu virus into the cells. The highest suppression activity is shown by the herb of wild thyme, reducing the titer of A_2 type flu virus by 3.5 log, whereas the weakest effect was observed for the decoction of herb of rupture wort. The absorption of B type flu virus was suppressed only by the decoction of flower of common mullein reducing the virus titer by 3 log.

Initial stages of replication of the A_2 type flu virus were influenced by decoctions of flower of primrose and root of common comfrey. They reduced the virus titer by 2.4 log and 2 log respectively. The decoction of flower of common mullein had a suppressive effect on the type B flu virus, reducing the virus titer by 2.5 log.

The following decoctions influenced the later developmental stages of the A_2 type flu virus: Septosan mixture and flower of primrose, reducing the virus titer by 3.8 log and 3 log respectively.

The B type flu virus was suppressed by the decoction of flower of yarrow the presence of which reduced the virus titer by 2.1 log.

Investigations of the preventive effects of the preparations on the cell cultures revealed that the garlic juice significantly reduced the susceptibility of the cells to both viruses. The virus titer of the A_2 type flu virus decreased by 4.2 log and of the B type virus by 3.65 log. The preventive effect on infections induced by the type A_2 flu virus was manifested to a lesser extent for the decoction of herb of common thyme and root of soapwort.

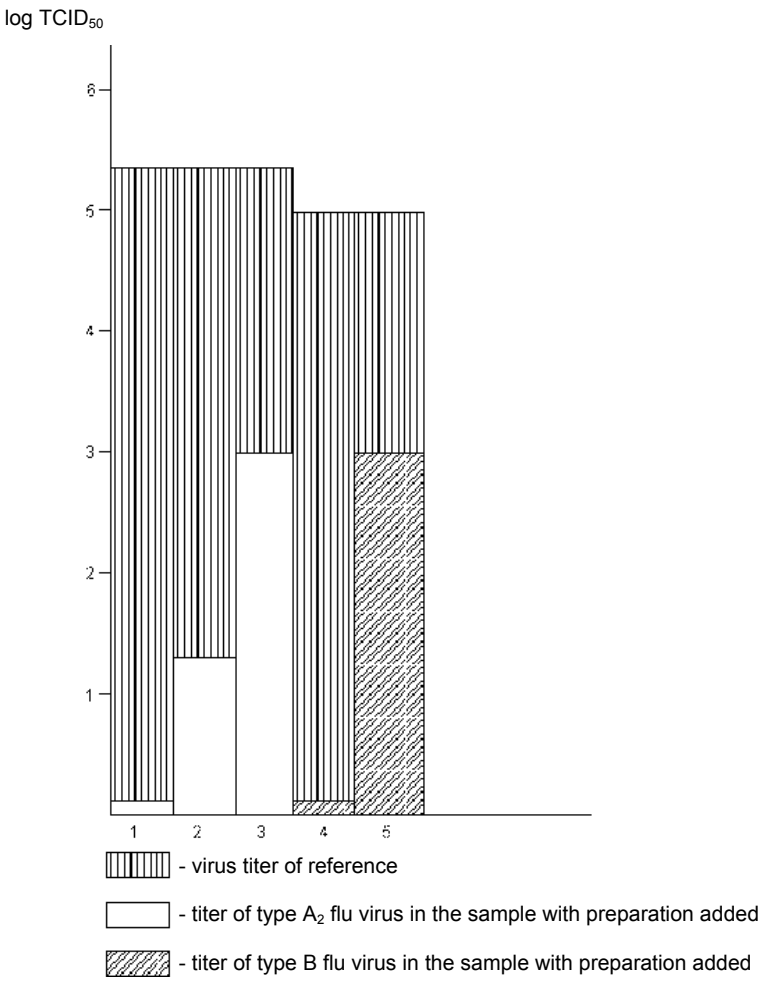

Fig. 2 Virucidal effect of decoctions
1 – Dec. Flos Verbasci
2 – Dec. Hb. Hyperici
4 – Dec. Flos Primuli
5 – Dec. Hb. Serpylli

Investigations of the virucidal effects of herbal preparations showed complete inactivation of the A_2 type flu virus under the influence of the decoction of flower of common mullein (Fig. 2). Inactivation of B type flu virus was also observed for decoction of flower of primrose.

Of 3 investigated preparations 2 showed preventive effects on the flu viruses in chicken embryos. Garlic juice (*Allium sativum*) reduced the virus titer of the A_2 type flu virus by 3.85 log and by 3 log in the case of B type virus. The decoction of herb of common thyme (*Hb. Thymi*) reduced the virus titer only with respect to the type A_2 flu virus by 2.5 log. Effects on the hemagglutination titer of type A_2 flu virus were observed for the decoctions of root of soapwort (*Rdx. Saponariae*) and herb of common thyme. They reduced the HA titer 7 times and 4 times respectively.

Suppressive effects on the replication of the A_2 type flu virus by simultaneous injection of the preparation and the virus into the chicken embryos were observed for decoctions of root of common comfrey (*Rdx. Symphyti*), herb of St. John´s wort (*Hb. Hyperici*), flower of primrose (*Flos Primuli*) and flower of common mullein (*Flos Verbasci)*. They reduced the virus titers by 3.5 log up to 1.8.

The titer of the B type flu virus was reduced by 2.5 log under the influence of the decoctions of flower of mullein and flowers of yarrow.

The reduction of the hemagglutination titer was observed only for the A_2 type flu virus in the presence of decoction of root of common comfrey, and to a lesser extent in the presence of herb of common thyme.

The suppressive effects on the intracellular replication of the A_2 type flu virus in chicken embryos was determined for the decoctions of flower of common mullein, root of common comfrey, herb of St. John´s wort and flower of yarrow (*Flos Millefolii*). They reduced the virus titer by 4.0 log up to 1.5 log. Activity against the B type flu virus was observed only in the case of the decoction of flower of mullein. In the presence of this preparation the virus titer was reduced by 4.7 log. Furthermore, the reduction of the hemagglutination titer for the A_2 type flu virus was observed in the presence of the decoctions of root of common comfrey, herb of rupture wort and root of soapwort.

Table I. Investigation of virucidal effects of decoctions on chicken embryos

Decoction	Flu virus	Virus titer ($TCID_{50}$)		HA titer (mean value)	
		Sample	Reference	Sample	Reference
Flos Verbasci	A_2	0	$10^{-5.55}$	0	1 : 258
Hb. Hyperici	A_2	$10^{-1.15}$	$10^{-5.35}$	0	1 : 382
Flos Primulae	A_2	$10^{-3.0}$	$10^{-5.35}$	1 : 8	1 : 430
Flos Primulae	B	0	$10^{-4.95}$	0	1 : 256
Flos Serpylli	B	$10^{-3.00}$	$10^{-4.95}$	1 : 160	1 : 199

The complete inactivation of the A_2 type flu virus following the direct contact of the virus with the preparation was observed for the decoctions of flower of mullein and herb of St. John´s wort, whereas the decoction of flower of primrose manifested a virucidal effect on the B type flu virus.

DISCUSSION OF RESULTS

The experimental results confirm the reports of numerous researchers who have argued that herbal preparations can be sources of antiviral agents. In this paper the antiviral activity of decoctions was confirmed for the preparations used quite often in the practice of the herbal therapy.

The activity of these decoctions can be multidirectional, as they can act preventively on host cells as in case of garlic juice, decoctions of herb of thyme and root of soapwort. *Ajzienmann* and *Dieriebiencowa*, *Fischer* (4-6, 28) in their investigations on preparations of sage and thyme have demonstrated the positive influence of the herb applied prior to the introduction of the flu virus into tissue culture or chicken embryos.

Badaiew (16), who has investigated the preparations of blue gum tree, has shown that the extract and the alcoholic tincture introduced prior to the virus injection saved 81.7% of chicken embryos from fatal infections with the Newcastle disease virus.

There are also findings demonstrating suppressive effects of decoctions on the adsorption stage of viruses on the cell. One can speculate that the mucous agents that make their way into the decoction in large amounts settle on the cell surface and mask or destroy the cell receptors. *Manolowa et al.* (1-2) investigating the coating agents have reported that the extract of common comfrey suppresses the adsorption of flu viruses in the tissue culture; however, it has no influence on the intracellular replication of viruses.

Besides their preventive and suppressive effects on the intracellular replication of viruses the herbal preparations also showed direct virucidal effects. Complete inactivation of the type A_2 flu virus was reported for the decoctions of mullein, and the inactivation of the type B flu virus was observed in the presence of the decoction of flower of primrose.

The virucidal activity of herbal preparations has also been confirmed by other investigators including *Bogdanowa* (29), Ukrainian researchers (30), *Ajzienmann* (4) and *Tippelt* (3).

CONCLUSIONS

Of 26 herbal preparations investigated, 12 suppressed the propagation of flu viruses in cell cultures and chicken embryos.

The preparations under test manifested preventive activity with regard to the cells susceptible to the virus or showed suppressive effects on the development of viruses in various stages of the intracellular replication or even a virucidal activity in course of *in vitro* tests.

The type A_2 flu virus displayed greater susceptibility to investigated preparations than the type B flu virus.

The comparison of results obtained during tests carried out on cell cultures and chicken embryos revealed stronger antiviral activity of herbal preparations with respect to the cell cultures.

The strongest suppressive influence on flu virus both in cell cultures and chicken embryos was reported for the decoction of flower of mullein.

Summary

Twenty six plant preparations were tested in respect of their antiviral activity against influenza viruses A_2 and B in cultures of chicken fibroblasts and in chicken embryos. Twelve preparations were found to display antiviral activity in fibroblast cultures, and only four in cultures of the viruses in chicken embryos. The antiviral effects were noted at different stages of virus propagation. The viruses A_2 and B were found to differ in sensitivity to particular preparations investigated. Decoction of the mullein (*Verbascum thapsus*) flowers was found to display the strongest antiviral effect.

T. варек

ВЛИЯНИЕ ПРЕПАРАТОВ РАСТИТЕЛЬНОГО ПРОИСХОЖДЕНИЯ НА РАЗВИТИЕ ВИРУСОВ ГРИППА

I. Действие растительных препаратов на развитие вирусов гриппа в культуре фибробластов куриных зародышей и в куриных зародышах

Резюме

Исследовали 26 растительных препаратов с точки зрения их действия против вирусов гриппа A_2 и B в культуре фибробластов куриных зародышей и в куриных зародышах. Установили противовирусное действие 12 препаратов в тканевой культуре и 4 препаратов в куриных зародышах.

Препараты оказывали тормозящее влияние на развитие вируса на разных стадиях накопления. Исследуемые вирусы обнаруживали неодинакую чувствительность по отношению к исследуемым препаратам.

Отвар из цветов коровяка оказывал наиболее сильное противовирусное действие.

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