ANTIVIRAL ACTIVITIES OF EXTRACTS OF THE LEMON BALM PLANT*

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Lemon balm *(Melissa officinalis)*, also called garden or bee balm, is a plant that was mentioned in the Bible as having medicinal properties. Hippocrates mentioned its use as a medicament,[1] and several medicinal properties attributed to lemon balm were described by Dioscorides in the first century A.D.[2] Even today aqueous extracts of lemon balm are used orally or, less often, applied externally.[1] Lemon balm contains, among other plant constituents, ethereal oils reported to be antibacterial,[3] and tannin.[4]

Extracts of various plants[5,6] and commercial tannic acid[7] have been described as being active against influenza A (PR-8) virus in eggs. Although previously reported to be inactive against influenza A virus and against a bacteriophage,[5] recent studies indicated that extracts of lemon balm possessed substantial antiviral activity against several viruses in eggs.[8] This activity has been further investigated in embryonated chick eggs, tissue culture, and hemagglutination test systems in an attempt to characterize the active principle of lemon balm and to explain its mode of action.

Experimental Methods and Results

Preparation of extract. One hundred grams of chopped lemon balm leaves (Meer Corp., New York, N. Y.) were added to one liter of distilled water. The leaves were soaked for one hour and then boiled vigorously for 30 minutes. The resulting dark brown aqueous extract was filtered through several layers of cheesecloth and clarified by centrifugation at 1,800 rpm for 20 minutes. Antibiotics were added to suppress bacterial and mold growth and the extract was stored at 4° C. The average content of solids in such aqueous extracts was 32 mg./ml.

Antiviral studies in eggs. Lemon balm extract diluted in water was injected via the allantoic sac into 9- to 11-day-old (white Leghorn) embryonated eggs at various intervals before and after virus infection. Newcastle disease (11914, egg passage); vaccinia (WR, mouse brain passage); herpes simplex (HF, mouse brain passage); and Semliki Forest (mouse brain passage) viruses* were diluted in tryptose phosphate broth (Difco) and injected by the same route. Extracts of lemon balm showed striking antiviral activity against these four test viruses (TABLE 1). Eggs were protected when injected with extracts as early as 72 hours before infection with Newcastle disease virus (ND). With vaccinia or herpes simplex virus, lemon balm extract was active when administered before or several hours after challenge with virus. Lemon balm extract did not protect eggs from a lethal injection of egg-adapted strains of influenza A (PR-8) or influenza B (Great Lakes strain) virus.†

Test for virucidal activity. Mixtures of equal volumes (4 ml.) of virus suspension and undiluted aqueous lemon balm extract were kept for four hours at 36° C.; for controls, virus only was suspended in tryptose phosphate broth. Each mixture then was diluted, in tenfold increments, in tryptose phosphate broth. Plaque counts of ND, vaccinia, herpes simplex, or Semliki Forest viruses that had been

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† All viruses were obtained from the American Type Culture Collection.

in contact with lemon balm extract agreed closely with those from corresponding virus controls. In this test, lemon balm had only a minimal neutralizing effect on influenza A or influenza B virus lethality for eggs. When the extract was injected into eggs before challenge with influenza A or B virus, no antiviral activity was detectable.

Hemagglutination studies. Under certain conditions, lemon balm extract was found to inhibit agglutination of erythrocytes by ND and mumps viruses. Determination of hemagglutination was carried out according to the microtechnique

TABLE 1

ANTIVIRAL ACTIVITY* OF LEMON BALM EXTRACT IN EGGS

Virus; dose	Extract injection†	Extract dose (mg./egg)				
		9.6	4.8	2.4	1.2	0.0
Newcastle; $100 \times LD_{50}$	−72	5/7	—	—	—	0/10
	−48	12/15	2/6	1/6	0/6	0/16
	−24	23/23	4/6	2/6	1/5	0/23
	− 6	7/7	6/6	4/7	1/7	0/7
	− 3	21/24	83/96	60/96	26/92	0/79
	0	12/12	10/12	7/12	1/13	0/13
	+ 2	0/16	—	—	—	0/16
Vaccinia; $100 \times LD_{50}$	−48	2/3	1/5	1/6	0/7	0/6
	−24	6/11	3/12	2/13	0/12	0/12
	− 6	3/5	2/7	1/7	0/7	0/6
	− 3	12/15	9/13	3/12	2/11	0/16
	0	5/7	3/5	1/6	0/5	0/7
	+ 3	4/5	3/6	0/5	0/6	0/6
	+14	0/9	—	—	—	0/10
Herpes simplex; $100 \times LD_{50}$	−24	2/4	1/5	—	—	0/5
	− 6	5/5	4/5	3/5	3/5	0/5
	− 3	5/5	2/5	1/5	—	0/5
	0	5/5	4/5	2/5	2/5	0/5
	+ 6	2/4	2/5	0/5	—	0/5
Semliki Forest; $10 \times LD_{50}$	−24	8/13	5/12	6/12	6/12	3/11
	−12	10/15	10/14	7/15	7/15	3/11
	− 6	8/14	8/15	5/14	2/14	3/11
	− 3	7/14	6/14	5/15	2/11	3/11
	0	10/14	10/14	6/14	4/14	3/11
	+ 1	9/13	5/13	2/14	2/13	3/11

* Expressed as number of survivors/total eggs.
† Expressed as hours before (−) or after (+) virus infection.

described by Sever[9] with 0.025 ml. of test and of control substances. Phosphate buffer (0.02 M) mixed with saline (*p*H 7.2) was used as a diluent. Virus preparations (infected allantoic fluid) were diluted in twofold steps starting with undiluted pooled material. The hemagglutination reaction was allowed to proceed for 30 minutes at 25° C. The highest dilution of virus found to agglutinate at least one-half of the erythrocytes (2+) was taken as one hemagglutinating unit.

In the hemagglutination-inhibition (HI) experiments, serial twofold dilutions of lemon balm extract were made. Four to eight hemagglutinating units of virus were added to all dilutions of lemon balm extract. The mixtures were incubated for 15 minutes at 25°C. and then erythrocytes were added. The reaction was read after further incubation for 30 minutes at 25° C. The results revealed that a 1:128 dilution of lemon balm extract inhibited hemagglutination of chick or guinea pig erythrocytes by ND and mumps viruses. In other experiments it was found that a 1:2 dilution of extract inhibited 256 hemagglutinating units of ND. Hemagglutination of erythrocytes by influenza A or influenza B virus was not affected by lemon balm.

Experiments were carried out to study reversal of the HI activity of the lemon balm extract. Suspensions of ND (eight hemagglutinating units) were added to equal volumes of aqueous lemon balm extract (1:2). The mixtures were incubated for 15 minutes at 25° C. and then one per cent gelatin was added with gentle mixing (the significance of the gelatin will be discussed subsequently). After addition of erythrocytes and incubation for 30 minutes at 25° C., it was found that gelatin could reverse the HI activity of the extract. These data suggest that the inhibitory action of lemon balm extract involves neutralization of virus. Indeed, when equal volumes of lemon balm extract (16 mg./ml.) and ND were incubated for 15 minutes at 25° C. and the mixture subsequently diluted 1:16 to 1:64, hemagglutination activity was restored (TABLE 2). Thus, it appears that lemon balm extract acts directly on the virus, resulting in the formation of a nonactive complex from which active virus is released by treatments that result in dissociation.

Effect on virus multiplication. In order to gain more information on antiviral activity *in vivo*, eggs were injected as previously described with various concentrations of lemon balm extract three hours before injection of ND virus. Subsequently, allantoic fluid was harvested, pooled, and tested for hemagglutination of erythrocytes and for infectivity in eggs. After 48 hours' incubation, virus was

TABLE 2

EFFECT OF DILUTING MIXTURES OF LEMON BALM EXTRACT AND NEWCASTLE DISEASE VIRUS

Mixture*		Hemagglutination†					
Extract (mg.)	Newcastle (hemagglutinating units)	Mixture diluted					
		1/2	1/4	1/8	1/16	1/32	1/64
16	5120	0	0	0	1+	2+	4+
4	5120	0	0	4+	4+	4+	4+
0.2	5120	4+	4+	4+	4+	4+	4+
0	5120	4+	4+	4+	4+	4+	4+

* Mixture was 1 ml. in total volume; buffered saline replaced extract in fourth set.
† 0 = no hemagglutination; 1+ to 4+ = various degrees of positive hemagglutination.

TABLE 3

EFFECT OF LEMON BALM EXTRACT ON MUPLTIPLICATION OF NEWCASTLE DISEASE VIRUS* IN EGGS

Extract† (mg./egg)	Titer of pooled allantoic fluids (6 eggs)‡	
	Hemagglutinin units/0.025 ml.	Egg infectivity (negative log)
9.6	0	<— 1
4.8	0	— 5.5
1.2	128	— 8.1
0.6	256	— 9.3
0.0	1024	—10.0

* Virus inoculum = 100 × LD_{50} injected via allantoic sac of nine-day-old embryonated chick eggs.

† Injected via allantoic sac three hours before injection of virus.

‡ Determined after incubation for 48 hours.

not detected in eggs treated with 9.6 mg. of extract; even 0.6 mg. of extract suppressed virus multiplication as compared to untreated controls (TABLE 3).

Modified extraction procedure. Lemon balm contains about five per cent tannin identified as a trimer of caffeic acid (3,4-dihydroxycinnamic acid) with a molecular weight of approximately 465.[4] Tannins and a very few plant polyphenols are precipitated by gelatin and by lead acetate; preliminary studies indicated that the active principle of lemon balm could also be precipitated by these reagents. Furthermore, gelatin eliminated the antiviral activity in tests in eggs (TABLE 4). The active principle could be precipitated by gelatin and then freed from the precipitate by digestion with 0.05 per cent trypsin at 36° C.[8] There is some degree of specificity in this reaction because no such precipitation was produced by bovine serum.

Attempts were made to obtain a semipurified material from lemon balm extracts by using lead acetate in a technique that was a modification of one used by Vuataz and co-workers[10] for separating plant polyphenols. Pigments were removed from 800 ml. of aqueous lemon balm extract by several extractions with chloroform, and residual chloroform was removed under vacuum in a rotating evaporator. The remaining aqueous phase was then extracted five times with equal volumes of ethyl acetate to remove some of the polyphenols, and residual ethyl acetate was removed by evaporation under vacuum. Saturated lead acetate was added dropwise to the aqueous phase. The precipitate was recovered by centrifugation (1,800 rpm for 20 minutes), washed five times with water (300-ml. portions), and resuspended in water to a final volume of 400 ml. The lead ions were removed with Dowex (AG-50W-X) resin (H-form, Calbiochem), leaving the active antiviral material in solution. The mixture was filtered and the resin was washed thoroughly with 20 per cent ethanol. The filtrates were combined, concentrated to one-third volume by evaporation under vacuum, and then freeze-dried to yield 5.1 gm. of a brown spongy powder. An aqueous solution of this semipurified material showed a two- to fourfold increase in protective activity when tested against ND in eggs.

Undiluted lemon balm extract (0.3 ml.) showed some toxicity in eggs. Although purification by gelatin or lead acetate precipitation removed some of this toxicity, it was thought that true toxicity should be investigated only when pure

TABLE 4

EFFECT OF GELATIN ON ANTIVIRAL ACTIVITY OF LEMON BALM EXTRACT

Virus; dose	Injections*			Survivors per total eggs
	Extract, 4.6 mg./egg	10% gelatin, 0.3 ml.	Distilled H_2O, 0.3 ml.	
Newcastle; 100 × LD_{50}	−24	− 3		3/25
	−24	+ 3		0/9
	−24	+24		0/9
	−24		+3	7/9
Vaccinia; 100 × LD_{50}	−24	− 3		0/8
	−24	+ 3		1/9
	−24	+24		1/9
	−24		+3	6/9
Herpes simplex; 100 × LD_{50}	−24	− 7		0/9
	− 7	−24		2/9
	−24		−7	6/10
Semliki Forest; 10 × LD_{50}	−24	− 3		1/9
	−24	+ 3		1/9
	−24	+24		1/9
	−24		+3	6/10
Control		− 3	+3	10/10

* Hours before (−) or after (+) virus infection.

material was available. It appears that the crude aqueous extracts are not extremely toxic to animals, since mice survived a 10-mg. dose injected subcutaneously daily for five days.

Tissue culture experiments. Semipurified extract was used in these experiments because of reduced toxicity for cell cultures. The material was dissolved in water at a concentration of 32 mg./ml. and adjusted to *p*H 6.7 with 1 N NaOH. This stock solution was further diluted with tryptose phosphate broth to obtain the desired concentrations. Antiviral activity was determined by the plaque technique in chick embryo fibroblast (CEF) monolayers. Experiments were done in the following ways: (1) exposure of monolayers to the extract before virus infection; (2) addition of the extract simultaneously with virus to the monolayers during virus adsorption; and (3) suppression of plaques by the antibiotic-disc technique.[11] All experiments were repeated two or three times.

Primary CEF monolayers were prepared, in 60-mm. plastic petri plates (Falcon, tissue culture type), from 11-day-old chick embryos. Growth medium consisted of Earle's balanced salt solution (BSS) containing 0.5 per cent lactalbumin hydrolysate, 2 per cent inactivated calf serum, 1 M tris(hydroxymethyl)aminomethane buffer at *p*H 7.6[12] (1.6 ml./100 ml.), 7.5 per cent sodium bicarbonate (1 ml./100 ml.), and penicillin and streptomycin (100 μg./ml.). A 6-ml. inoculum (4 × 10^6 cells/ml.) was added to each plate. Cultures were obtained within three days after seeding, during which period confluent monolayers were formed.

Unless otherwise stated, plaque counts were determined by aspirating the growth medium and infecting the monolayers with 0.5 ml. of a virus suspension diluted in tryptose phosphate broth to contain 100 plaque-forming units (PFU) per 0.5 ml. After incubation at 36° C. for one hour, unadsorbed virus was removed by washing the monolayers twice with BSS. The monolayers were overlaid with 6 ml. of one per cent agar medium,[13] (but without phenol red and gelatin). After four days' incubation at 36° C. (two days with Semliki Forest virus), the monolayers were vitally stained with 0.11 per cent iodonitrotetrazolium chloride.[11] The results were measured as the percentage of plaque-forming units (PFU) in treated plates compared to untreated plates.

When CEF monolayers were treated with various concentrations of semi-purified extract before virus infection, the antiviral effect increased with increasing dose of extract (TABLE 5). ND appeared to be more susceptible than Semliki Forest virus. Control plates received tryptose phosphate broth (0.5 ml.) instead of extract. In similar experiments, the addition of gelatin to extract-treated monolayers did not appear to counteract the inhibition of virus plaque formation. We have no explanation for this apparent disagreement with results in eggs. The extract did not seem to injure CEF monolayers at the concentrations and time of exposure employed.

In the next series of experiments, cell monolayers were exposed to extract and virus simultaneously; infectivity of the four susceptible viruses previously tested in eggs was inhibited. Plaque production by ND, herpes simplex, and Semliki Forest viruses was all but eliminated when the virus was mixed with increasing amounts of extract, and substantial antiviral activity was still detectable against ND, vaccinia, and herpes simplex viruses with doses of extract as low as 0.3 mg. (TABLE 6). For reasons not altogether clear, it has not yet been possible to show antiviral activity in mammalian cell cultures.

In the antibiotic disc-plaque inhibition experiments, ¼-inch antibiotic discs were impregnated with lemon balm extract. Monolayers of cells were infected with 2 to 7 × 10^3 virus PFU and overlaid with agar medium as before. The discs

TABLE 5

INHIBITION OF VIRUS PLAQUE FORMATION BY PRETREATING CHICK CELL MONOLAYERS WITH LEMON BALM EXTRACT*

Extract (mg.)	Plaque-forming units as per cent of control	
	Newcastle disease	Semliki Forest
8.0	4	21
4.0	6	36
2.0	14	57
1.0	40	—
0.5	56	88
0.3	89	—
0.0 (control)	100	100

* Monolayers were pretreated with 0.5 ml. of extract solution for one hour at 36°C. Lemon balm extract was removed and monolayers were washed twice with BSS; 0.5 ml. of virus suspension (100 PFU) was added for one hour at 36° C. Virus was removed and cell monolayer was washed twice with BSS and then overlaid with agar medium.

TABLE 6

INHIBITION OF VIRUS PLAQUE FORMATION BY SIMULTANEOUS ADDITION OF LEMON BALM EXTRACT AND VIRUS TO CHICK CELL MONOLAYER*

Extract (mg.)	Plaque-forming units as per cent of control			
	Newcastle disease	Vaccinia	Herpes simplex	Semliki Forest
4.0	1	50	<1	2
2.0	5	—	20	7
1.0	15	58	46	22
0.5	32	—	—	74
0.3	46	66	63	100
0.1	89	96	—	—
0.0 (control)	100	100	100	100

* A mixture of virus (100 PFU) and lemon balm extract in 0.5 ml. was added to cell monolayer for one hour at 36°C. After mixture was removed, the cell monolayer was washed twice with BSS and then overlaid with agar medium.

were placed on the hardened agar surface. The cell sheets were stained as previously described. The results showed plaque-free zones averaging 21 mm. against ND, 23 mm. against vaccinia, 16 mm. against Semliki Forest, and 28 mm. against herpes simplex viruses.

Qualitative analysis of antiviral principle. The following chemical reactions, generally characteristic of tannins, were given by aqueous lemon balm extract. The extract formed a precipitate with gelatin and with 10 per cent egg albumin. The activity was removed from solution by treatment with hide powder (the supernate was inactive when tested against ND in the HI test). Lemon balm extract gave a dark bluish green color with one per cent ferric sulfate solution and gave a precipitate with lead salts, with quinine sulfate, and with the organic base antipyrine (phenazone). Lyophilized lemon balm extract was soluble in water and in methanol, slightly soluble in 95 per cent ethanol, and insoluble in chloroform, ethyl acetate, and acetone. With one exception, all of these reactions were also shown by active material freed from gelatin by trypsin digestion and by active material freed from lead by ion exchange with resin. The active agent freed from gelatin could not be reprecipitated with fresh gelatin. This result suggested that the active sites for gelatin attachment were still occupied by peptides, but this did not seem to inhibit the antiviral activity of this preparation.

Discussion

It appears from these studies that the antiviral activity is indeed due to the tannin described by Karl Herrmann.[4]

A satisfactory explanation of the antiviral activity of lemon balm extract involves consideration of the complex nature of the cell surface and of the virus. The hemagglutination-inhibition data show that the extract neutralized certain viruses on contact. Interaction between viruses and tannins is thought to be due to hydrogen bonding and results in precipitation of the virus from suspension.[14] The active principle of lemon balm extract, behaving like antibody, attached to

the virus and thus prevented union between it and the cell receptors. Nevertheless, the weak bond between the virus and the active principle could be dissociated by gelatin or by dilution of the virus-lemon balm extract mixture. It has been suggested by others[15] that the main reaction between gelatin and tannins seems to involve combination of the amino groups and the peptide groups of gelatin with the free hydroxyl groups of the tannin to form intermolecular compounds. Studies of interactions between lemon balm tannin and viruses might, therefore, give information on the nature of the protein moiety of viruses.

Although lemon balm extract neutralized certain viruses, not all of its antiviral activity can be explained on this basis alone. From experimental data obtained from studies of eggs and of tissue culture, it is proposed that some of the antiviral activity of lemon balm extract depends on a reaction of the active principle with the surface of the host cell. Since gelatin reversed the protective activity of the extract in eggs, it is conceivable that the active principle could have an affinity for other proteins involving the cell surface. The site of attachment of the active principle on the host cell does not seem to involve myxovirus receptors since the extract was not antiviral against influenza A or B virus in eggs.

The chemical effects of tannic acid on tissues have been described by other investigators. It is known that tannic acid reacts with proteins to form a material that is more resistant to degradation by enzymes.[16] This observation suggests that combination of tannins with tissues could induce resistance in these tissues to virus infection. Olitsky and Sabin[17] reported that intranasal instillation of tannic acid protected primates from infection with equine encephalomyelitis and poliomyelitis subsequently instilled by the same route. The authors stated that the tannic acid seemed to exert its effect on the nasal mucosa of the host rather than on the virus.

Summary

Aqueous extracts of lemon balm (*Melissa officinalis*) protected embryonated eggs when injected before lethal challenge with Newcastle disease (ND), vaccinia, herpes simplex, or Semliki Forest virus. Gelatin injected into eggs after lemon balm extract eliminated the antiviral activity. Extracts of lemon balm suppressed infectivity of the four susceptible viruses in chick embryo fibroblast monolayers. Hemagglutination of erythrocytes by ND and mumps viruses was inhibited by lemon balm extract, but hemagglutination activity could be restored by addition of gelatin or by dilution of nonactive mixtures of virus and extract. The active principle of lemon balm extract was precipitated by gelatin and by lead acetate. The recovery of antiviral activity from these precipitates and the qualitative chemical data suggest that the active moiety is a tannin. The mode of action of the tannin seems to involve the surface of the host cell in addition to neutralization of the virus.

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