ECHINACEA PURPUREA L.

INFLUENCE OF *CUCUMBER MOSAIC VIRUS* (CMV) ON THE MOTHER TINCTURE

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After having examined in two previous articles the influence of viral infections on the quantity and quality of essential oils extracted from thyme (*Thymus vulgaris* L.) and from clary sage (*Salvia sclarea* L.), it is now the turn of *Echinacea purpurea* (L.) Moench. (Bellardi *et al.*, 2001 a, b). This is certainly one of the most widely used medicinal species used in pharmacology thanks to its multiple therapeutic uses: prophylaxis and treatment of colds, ulcers, wounds, burns, aphthas and dermatitis. For this purpose, not only the aerial part is used but also the hypogean part (roots and rhizome), from which, in the fresh state. the Mother Tincture is obtained (M.T.). Unlike the previous two analyses, the comparison has been made not between the oils, but between the M.T. extracted from *E. purpurea* plants infected by the cucumber mosaic virus (CMV) and healthy ones. Below are illustrated the procedures of the survey followed, beginning with the identification in the field of the disease caused by CMV, continuing with the isolation and identification of the pathogen to the analytical comparison (gas-chromatography-mass spectometry) of the M.T. obtained from the "infected" and "healthy" roots.

VIROLOGY STUDY

The samples of *E. purpurea* to be analysed were collected during visits in the summer-autumn of 1999 to the Giardino delle Erbe (Herb Garden), Casola Valsenio (Ravenna). An accurate visual examination of the medicinal herb being cultivated allowed the observation of a symptomatology referable to the presence of the virus on the leaves and flowers (Fig. 1).
In particular, on almost all the plants (more than 90%) mosaics, variegation and yellow arabesques were observed on the leaves, which were often malformed and with bullas (Fig. 2). The petals of the flowers, of reduced dimensions, showed contractions, deformations and speckling (Fig. 3). The plants with these symptoms appeared smaller and with a bushy appearance compared to asymptomatic plants (Fig. 4). Leaf samples were collected from plants with and without symptoms in order to ascertain whether the virus was present or not.
The routine techniques were applied for the virology tests: biological tests (mechanical inoculations on indicator herbaceous plants), observations under the electronic microscope ("*leaf-dip*") and serum diagnosis such as ISEM (*immunosorbent electron microscopy*: immunosorbent electronic microscopy), GLAD (*gold-labelled antibody decoration*) and PAS-ELISA (*protein A sandwich-enzyme-linked immunosorbent assay*).
The mechanical inoculations carried out using leaf juice from the symptomatic samples of *E. purpurea* have induced, on species belonging to the family of the *Chenopodiaceae* (*Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *C. murale* L., *C. album* L.), local symptoms consisting of a chloronecrotic speckling. After the first inoculations on these *Chenopodiaceae*, 38 species, in 12 botanical families, were then inoculated. Of these, 15 contracted the infection, including some *Solanaceae* (*Nicotiana benthamiana* L., *N. occidentalis* L. and *N. tabacum* L. "Samsun" and "Xanthi") which showed local and systemic symptoms typically referable to infections from CMV. The inoculations carried out with asymptomatic samples gave a negative result in all cases. The observations under the electronic microscope of the "*leaf-dips*" prepared with leaf juice from the symptomatic and asymptomatic samples of *E. purpurea*, as well as of herbaceous plants infected artificially, did not reveal the presence of viral particles of the filamentous type.
The application of the ISEM technique using specific antiserums of isodiametrical viruses has allowed hypothesizing the presence of a stock of CMV. In fact, using the anti-CMV serum, it was possible to observe in the individual preparations isodiametrical viral particles "decorated" with the specific antibodies of this *Cucumovirus* (Fig. 5). The application of the GLAD technique also allowed observation (again under the electronic microscope) of isodiametrical viral particles uniformly limited by granules of colloidal gold, only after incubation with the anti-CMV serum. The immunoenzymatic PAS-ELISA tests have allowed the definite identification of the virus found in the plants (epigean part) of symptomatic *E. purpurea* as an isolated CMV. Once *E. purpurea* plants in the field hosting CMV ("infected" sample) and a sample free of this virus ("healthy" sample) were identified, before extraction of the M.T. from their roots, they were subjected to further virological tests applying some of the techniques mentioned above:: biological tests and PAS-ELISA. The virus was only present in the root tissue of the "infected" sample and not in that of the "healthy" sample.

PREPARATION OF THE MOTHER TINCTURE (M.T.)

The preparation of the mother tincture (M.T.) was carried out in March 2000, in the laboratory of the Giardino delle Erbe, using the "maceration" technique.
The fresh *E. pupurea* material belonged to two separate batches: the first included roots of "healthy" plants, the second those of plants "infected" by CMV. After thorough cleaning, the roots of the two batches were finely chopped (to make the operation of extracting the solvent easier) and left to macerate in glass recipients covered with a mixture consisting of 336 ml of distilled water and 664 ml of 65° ethyl alcohol, in a ration of 1: 10 (ratio in drug weight/solvent = 1:10).
The maceration (at room temperature) lasted for about 21 days, at the end of which the contents of the recipients were pressed and filtered on paper 3 times (Pedretti, 1990).
The tinctures obtained from the two batches of *E. purpurea* were measured for their degree of alcohol (using the appropriate alcoholometer) to record any variations, then placed in two recipients of dark glass ("healthy" M.T. and "infected"

- GC-MS system:
- *Trace* GC " 2000 series" connected to a *Thermo Quest* ion trap mass spectometer.
- Electron impact: 70 Ev.
- Column: Rtx®-5MS fused silica capillary column (Restek Corporation, Bellefonte, USA) . Dimensions: 30m x 0.25mm ID, film thickness 0.25µm, internally lined with Crossbond: 5% phenyl-95% dimethyl polysiloxane.
- GC-MS system: Parameters of the analysis:

GC: - injection chamber: injection in column with *Split/Splitless* technique (splitting ratio: 1: 50 and 1: 75):
injector temperature: 250°C.
volume of sample injected: 1ml.
transporting gas: helium (1ml/min.).
oven: programme for linear increase of temperature:
- initial T° : 50°C (for 5 min.)
- T° increase: 5°/min.
- final T° : 220°C.

MS: - temperature of *Transfer line:* 250 °C.
source of ions: 200 °C.

COMPOSITION OF THE LIPOPHILIC COMPONENTS. MOTHER TINCTURE OF E. PURPUREA (L.) MOENCH.

N°	R.T.	Constituents of the Mother Tincture (M.T.)	"healthy" M.T.	"infected" M.T.
1	23.14	B-Caryophyllene	0.65 %	0,30 %
2a	24.35	1,8-Pentadecadiene	0.50 %	1,28 %
2b	24.40	1,8,11-Pentadecatriene	0.23 %	0,63 %
3	24.69	Germacrene D	10.46 %	4,73 %
4	25.41	unidentified	0.77 %	0,83 %
5	35.57	Dodeca-2E, 4E-dien-1-yl-isovalerate	5.03 %	2,80 %
6	36.89	compound "4"	6.48 %	12,94 %
7	37.87	compound "2"	0.92 %	1,81 %
8	38.64	unidentified	13.86 %	9,30 %
9	38.78	compound "5"	7.00 %	5,42 %
10	39.19	compound "8" or "9"	26.80 %	23,73 %
11	39.63	compound "8" or "9"	9.76 %	12,42 %
12	40.74	compound "11"	4.67 %	6,86 %
13	41.93	compound "3"	5.55 %	6,87 %
14	45.06	unidentified	1.76 %	2,45 %
		others (unidentified)	5.56 %	7,62 %

Table 1

M.T.) and kept at room temperature until the chromatographic analysis.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS SYSTEM)

This analysis was carried out with the aim of assessing percentage differences in the quantity of the lipophilic constituents of the M.T. of the two batches of roots.
The data relative to the instrumentation used is as follows: Before proceeding with the introduction of the sample into the gas chromatograph, 1ml of the M.T. was extracted 3 times with 2ml of the hexane-acetate of ethyl mixture (in a ratio of 1:1) in a separator funnel.
The organic extracts were collected and concentrated to a volume of 1 ml, in order to obtain a gas-mass analysis that was sufficiently sensitive for the characterization of the individual components.
A microlitre of this solution was then injected into the GC-MS system, by the *Split/Splitless* technique under pre-established thermal conditions; the sample was transported by the gas along the chromatographic column to the detector, consisting of the source of ions of the mass spectometer (MS).
The percentage composition of the M.T. was calculated on the basis of the area of peaks of the chromatogram without a correction factor with the method of percentage areas, given by the ratio between the area of each individual peak and the sum of all the peak areas obtained.
The identity of the peaks was confirmed on the basis of the mass spectrums obtained and compared with those shown in literature (Operan *et al.*, 1998).
The gas chromatographic analysis of the two extracts ("healthy" M.T. and "infected" M.T.) has shown a general profile that is substantially similar where at least 14 main peaks were identified, corresponding to the same number of compounds for some of which, however, the identity is not known (tables 1 and 2). Making a comparison between the two chromatograms (diagram 1), difference in the extracts can be noticed as far as the intensity relative to some peaks is concerned, and in particular a difference of 5.73% for the compound with 24.69 retention time (R.T.), that is, Germacrene D (10.46% in the "healthy" specimen and 4.73% in the "infected" one).
Further quantitative differences can be found for other compounds such as: dodeca-2E, 4E-dien-1-yl-isovalerate (5.03% in the "healthy" sample and 2.80% in the "infected" sample) and dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide (7.00% in the "healthy" sample and 5.42% in the "infected" one), as shown in table 1. Vice versa, an increase of 6.46% can be noticed for the compound with 36.89 R.T. (undeca-2E, 4Z-diene-8, 10-diynoic acid 2-methylbutylamide), in a concentration of 6.48% in the "healthy" sample and 12.94% in the "infected" sample. However, the most significant variation remains that of Germacrene D.

THE COMPOUNDS SHOWN WITH THE NUMBERS CAN BE TRACED BACK TO THE CLASSIFICATION OF BAUER ET AL. (1988), AS FOLLOWS:

Compound "4":	undeca-2E, 4Z-diene-8, 10-diynoic acid 2-methylbutylamide
Compound "2":	undeca-2Z, 4E-diene-8, 10-diynoic acid isobutylamide
Compound "5":	dodeca-2E, 4E, 10E-trien-8-ynoic acid isobutylamide
Compound "8":	dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide
Compound "9":	dodeca-2E, 4E, 8Z, 10Z-tetraenoic acid isobutylamide
Compound "11":	dodeca-2E, 4E-dienoic acid isobutylamide
Compound "3":	dodeca-2E, 4Z-diene-8, 10-diynoic acid isobutylamide

Table 2

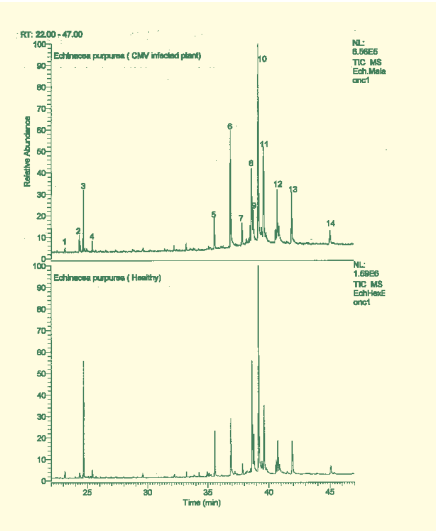

DIAGRAM 1
GC-MS Chromatogram relative to the "infected" (above) and "healthy" (below) Mother Tincture (M.T.) of E. purpurea (L.) Moench. The peaks are numbered in accordance with

The virological test carried out shows that the *E. purpurea* cultivated in the Giardino delle Erbe is infected by CMV, the cause of a very serious symptomatology which can compromise the yield, especially due to dwarfism (reduced growth) of the plants.
From the 1960's-70's, this species has been known in other parts of the world (especially in Germany) as the natural host of CMV and, as far as Italy is concerned, the first report dates back to 1997, when the virus was identified on this species, again in the Emilia-Romagna region (Bellardi *et al.*, 1997).
After having evaluated the constant association between the symptomatology shown in the field by leaves and flowers and CMV, the M.T. obtained from the roots of healthy and infected plants was then analysed in order to ascertain any differences in the composition. From the analysis of the chromatograms obtained, the difference appeared clear, especially of Germacrene D, which was present in quantities more than double in the "healthy" sample compared to the "infected" one. This significant reduction of Germacrene D in the plants infected by CMV takes on particular importance if this compound is considered as one of the main constituents of the plant to which it owes its immunostimulant action (Campanini, 1998). In fact, it contributes to the increase in the number and activities of the immunocompetent cells (macrophages and T lymphocytes), as well as having a healing (thanks to stimulation of the fibroblasts) and anti-inflammatory action.
As already observed regarding the oils extracted from thyme and clary sage, differences in composition referable to the presence or not of a virus in the plant also exist for the Mother Tincture obtained from *E. purpurea*. This should induce reflection on the need to consider with greater attention the state of health of medicinal species being cultivated, not only to reduce the quantitative damage (drop in yield) due to diseases caused by these pathogens, but also to improve the quantity and quality of the essences extracted from the epigean and hypogean parts of the plants. Further comparative studies and analyses on other medicinal species are naturally necessary to confirm the results obtained to date.

CONCLUSIONS

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