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The components of *Melissa officinalis* L. that influence protein biosynthesis in-vitro

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An investigation of an inhibiting activity of a substance(s) in a tanninless extract from *Melissa officinalis* leaves on protein biosynthesis in-vitro has been made. At least two components which inhibited protein biosynthesis were present in the extract; these were caffeic acid and an unidentified glycoside. Freshly prepared buffered solutions of caffeic acid inhibited protein biosynthesis less than solutions stored for several days at room temperature (20 °C). In this case derivatives of caffeic acid were formed, which may be responsible for the increase in the inhibitory effect of stored caffeic acid solution. An inhibitor, in the homogeneous state, was also isolated from the glycoside fraction of *M. officinalis*. Studies on the mechanism of the action of this inhibitor revealed its effect is to use the result of a direct interaction with elongation factor EF-2, and the blocking of the binding reaction of EF-2 with ribosomes.

From previous reports it is known that aqueous extracts of *Melissa officinalis* inhibit virus development (Herrman & Kucera 1967; Kucera & Herrman 1967; Skwarek 1979) and tumour cell division (Ożarowski 1980, 1982). Our preliminary experiments have shown that aqueous extracts of *Melissa officinalis* will inhibit protein biosynthesis in a cell-free system from liver cells, and that non-tannin substances are mainly responsible for this effect (Chlabicz et al 1984). We now describe the isolation and purification of this substance from *Melissa officinalis*.

MATERIALS AND METHODS

Materials

Folium *Melissae* (dried leaves) was purchased from "Herbapol", Poland. PCA (perchloric acid 60%, VEB Laborchemie, GDR); sucrose puris (BDH, UK), Tris (hydroxymethyl/aminomethane AH, 2-mercapto-ethanol, Fluka A.G., Buchs S.G.); GTP (guanosine-5′-triphosphate, Calbiochem); ATP (adenosine-5′-triphosphate, disodium salt, Koch Light Laboratories Ltd, UK); DTT (dithiothreitol, Loba-Chemie, Wien-Fischamend, Austria); PPO (2,5-diphenyloxazol, E. Merck); POPOP (*p*-bis(2)-5-phenyloxazolyl/benzene, scintillation grade, New England Nucl. Copr., Chem. Div.); L-[1-^{14}C]-Leucine(60 mCi mmol^{-1}, Amersham, UK); Glass fibre filters GF/A (Whatman Biochemicals Ltd, UK); dipotassium phosphate (Xenon, Poland).

Methods

For the preparation of a tanninless aqueous extract of *Melissa officinalis* leaves, 2 g was defatted with 50 ml of light petroleum (b.p.), then with 50 ml of chloroform and extracted with 50 ml of methanol. The solvent was evaporated and the residue extracted with 50 ml of Tris HCl buffer, pH 8·0. The tannins were removed from the buffered solution by extraction with ethyl acetate (5 × 50 ml). The complete removal of the tannins was checked by the specific chemical reactions described by Strzelecka & Kamińska (1977).

Isolation of glycoside fraction. Chlorophyll was extracted from 3 g of the dry leaves (Strzelecka et al 1982) with 4 × 50 ml chloroform which was then discarded. Warm distilled water (200 ml) was added to the residue and the mixture placed in a boiling water bath for 30 min. The extract was filtered and the residue washed with 50 ml of warm water and then refiltered. Filtrates were combined and 40 ml of an aqueous solution of basic lead acetate was added. Following sedimentation, 2 g of disodium phosphate was added to the mixture, which was then filtered. The filtrate was then adjusted with 6 M HCl to pH 5·0 and extracted with ethyl acetate (5 × 50 ml). This extract was concentrated under vacuum for investigation.

Preparative paper chromatography. Glycoside (10 ml) fractions obtained after extraction with ethyl acetate, were placed on 5 sheets of the Whatman No. 3 paper and chromatographed in solvent (acetic acid–water 15 : 85 v/v). Fluorescent spots were eluted with 250 ml of 0·05 M Tris HCl buffer pH 8·0 and the eluates were extracted with ethyl acetate (2 × 50 ml).

The extract was evaporated under vacuum and the

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dry residue dissolved in 10 ml of 0·05 M Tris HCl buffer, pH 8·0 and used for further investigation; 0·01 ml of this solution was equivalent to 0·4 mg of dry *Melissa* leaves.

The cell-free system from the rat liver or Guerin tumour cells, the ribosomes, [^{14}C]Leu-tRNA and elongation factors EF-1 and EF-2 were prepared according to Skogerson & Moldave (1967, 1968a, b), Gałasiński & Moldave (1969), Jabłonowska et al (1983), Kopacz-Jodczyk et al (1984).

Incorporation of [^{14}C]leucine into proteins in-vitro. A 1 ml amount of the incubation mixture contained: 500 μg of ribosomes, 68 mM Tris HCl (pH 8·0), 6 mM $MgCl_2$, 80 mM NH_4Cl, 0·2 mM GTP, 1 mM DTT, [^{14}C]Leu-tRNA (ca 4000 counts min^{-1}), 0·2 ml of the supernatant containing the elongation factors EF-1 and EF-2 or adequate amounts of purified EF-1 and EF-2 together with or without the extracted inhibitory substance from *Melissa officinalis*. Incubation was at 37 °C for 30 min, then an equal volume of 10% trichloracetic acid (TCA) was added and the mixture heated in a boiling water bath for 15 min. The protein sediment retained on the glass fibre filter GF/A was washed with 5% TCA and dried. The radioactivity retained on the filter was measured by scintillation counting (Isocap-300, Nuclear Chciago), and results corrected.

Inhibition of ribosome activity. A ribosome suspension of 0·4 ml (30–40 mg ml^{-1}) was incubated with 0·4 ml of inhibitor solution at 37 °C for 30 min. At the same time 0·4 ml of ribosomes and 0·4 ml of 0·05 M Tris HCl, pH 8·0, as a blank were incubated. Both samples were layered on a sucrose gradient consisting of two layers of 4 ml 1 M sucrose and 4 ml 0·5 M sucrose, then centrifuged for 4 h at 105 000 *g*. The ribosome pellet was washed and resuspended in the same buffer. The activity of these ribosomes was examined by the incorporation of [^{14}C]leucine into proteins as described above.

RESULTS AND DISCUSSION

Our preliminary experiments have shown that water extracts from *Melissa* leaves, inhibit protein biosynthesis of rat liver cell-free systems. Initially, the tanninless fraction was believed to be responsible for this inhibitory effect (Chlabicz et al 1984).

The glycoside fraction from *M. officinalis* was isolated and its effect on [^{14}C]leucine incorporation into proteins in-vitro was examined. The results in Table 1 show that the inhibitory effect of the glycoside fraction is similar to that of the total extract from *M. officinalis*. Some difference may arise from the losses of glycoside during the isolation.

Table 1. Influences of the total extract and glycoside fraction from *M. officinalis* on [^{14}C]leucine incorporation into proteins in-vitro.

Extract* added ml	[^{14}C]Leucine incorporated (counts min^{-1}) A	B	Inhibitory effect (%) A	B
—	1707	1707	—	—
0·04	76	468	95·6	72·7
0·06	8	460	99·5	73·1
0·08	1	454	100·0	73·4
0·10	—	320	100·0	81·3

A Total extract.
B Extract from PbS sediment (glycoside fraction).
* 0·01 ml of the extract was equivalent to 0·4 mg of dry leaves.

We have also found that caffeic acid, which is present in extracts from *M. officinalis*, inhibits protein biosynthesis in-vitro and this inhibitory effect is increased after storage of this acid in buffered solution (Table 2).

Chlorogenic acid, also present in the extract from *M. officinalis*, does not inhibit [^{14}C]leucine incorporation into proteins.

The glycoside fraction isolated from *Melissa* leaves was analysed by paper chromatography. This fraction consisted of two components. One of these ($R_F = 0{\cdot}64$), was responsible for the inhibitory effect of the glycoside fraction (Table 3).

The inhibitory component of the glycoside fraction was purified by paper preparative chromatography. Its homogeneity was examined by paper or thin-layer chromatography.

Fig. 1 shows a distinct effect of the inhibitor isolated from the glycoside fraction of *M. officinalis* on [^{14}C]leucine incorporation into proteins in-vitro. From these results we can conclude that this com-

Table 2. The effect of caffeic acid on [^{14}C]leucine incorporation into proteins in-vitro.

Caffeic acid added mg	Solution of freshly prepared caffeic acid* [^{14}C]Leucine incorporated (counts min^{-1})	Inhibitory effect (%)	Solution of caffeic acid stored for 48 h† [^{14}C]Leucine incorporated (counts min^{-1})	Inhibitory effect (%)
—	1842	—	2017	—
0·1	1706	7·0	1888	6·4
0·2	1637	11·2	950	54·9
0·3	1384	25·9	663	67·2
0·4	1278	30·6	330	83·7
0·5	822	55·4	203	90·0

* Caffeic acid solution was prepared in 0·05 M Tris buffer adjusted with HCl to pH 8·0.
† Buffered solution of caffeic acid was stored at room temperature.

Table 3. The effect of the glycoside fraction and its inhibitory component on the [^{14}C]leucine incorporation into proteins.

Fraction added* (ml)	[^{14}C]Leucine incorporated (counts min^{-1}) Glycoside fraction	Inhibitory component	Inhibitory effect (%) Glycoside fraction	Inhibitory component
—	3010	3010	—	—
0·02	2057		31·7	
0·04	1052	2389	65·1	20·7
0·6	643		78·7	
0·08	392	2170	87·0	27·9
0·10	286		90·5	
0·12		1613		46·4
0·16		1276		57·6
0·20		1174		61·0

* 0·01 ml of the glycoside fraction is equivalent to 0·4 mg of dry leaves. 0·01 ml of the eluate containing inhibitory component is equivalent to 0·2 mg of dry leaves.

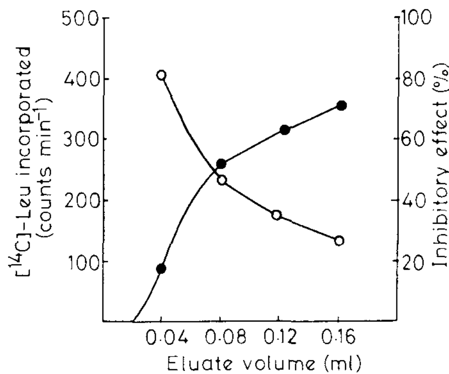

FIG. 1. The effect of the inhibitory component ** from the glycoside fraction of *M. officinalis* on [^{14}C]leucine incorporation into proteins in-vitro. ○——○ [^{14}C]leucine incorporated into proteins. ●——● Inhibitory effect. * 0·01 ml of eluate is equivalent to 0·4 mg of dry leaves, ** inhibitor which was purified by preparative chromatography.

pound is responsible for the inhibitory effect of the *Melissa* extract. Ribosomes, however, after preincubation with inhibitor, retained their activity and were not influenced by *Melissa* extracts.

The substance from *M. officinalis* inhibited the activity of the elongation factor EF-2 up to 80% (Fig. 2). This observation was checked by the method of Hobden (1978). On the basis of the results in Fig. 3 it can be concluded that the inhibiting substance from *Melissa* distinctly diminished the amount of peptidyl-tRNA in donor sites of ribosomes. This may be a result of the inhibition of peptidyltransferase activity or possibly the translocation step. Addition of puromycin to the incubation mixture caused a distinct decrease of [^{14}C]-leucine incorporation into the peptide. The formation of a peptide-puromycin complex was catalysed, as for peptide synthesis, by the enzyme

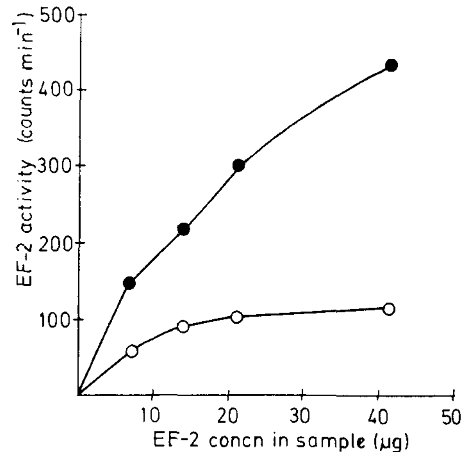

FIG. 2. The effect of inhibitory component from glycoside fraction of *M. officinalis* on EF-2 activity *. ○——○ EF-2 activity with no inhibitor added. ●——● EF-2 activity assayed in the presence of 0·16 ml of active inhibitive fraction eluted from the chromatogram. * EF-2 activity was assayed in the total elongation system and it is expressed as counts min^{-1} of [^{14}C]leucine incorporated into proteins in the presence of increasing amounts of EF-2 and a stable amount of EF-1.

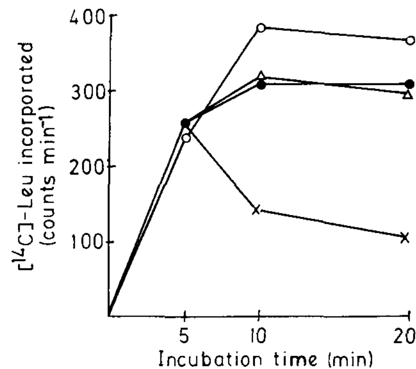

FIG. 3. The influence of puromycin and cycloheximide on the inhibitory substance from *M. officinalis*. The y-axis is [^{14}C]leucine incorporated into pept-tRNA. ○——○ without inhibitor. ●——● with inhibitor. ×——× with puromycin (0·1 ml of 10^{-5} M solution) which was added to the mixture after incubation with inhibitor for 5 min. △——△ with cycloheximide (0·1 ml of 1% solution) which was added to the mixture after incubation with inhibitor for 5 min. The experiment was essentially carried out according to Hobden (1978). The components of the test of [^{14}C]leucine incorporation into proteins (Gałasiński & Moldave 1969) were added to 4 sets of 3 tubes each. [^{14}C]Leucine-tRNA of 10 000 counts min^{-1} was added. The incubation at 37 °C was carried out for 5, 10 or 20 min. Enzymatic reaction was stopped by addition of 1 ml of 2% acetyltrimethylammonium bromide and 1 ml of acetate buffer, pH 5·5, containing 0·5 g of yeast RNA (as carrier). The mixtures were allowed to stand for 3 h, for better pellet aggregation, they were then filtered through glass fibre filter GF/A and washed with distilled water. The filters were immersed in 10% TCA and heated at 90 °C for 20 min to remove [^{14}C]leucine-tRNA. Filters were washed with 5% TCA and dried. Radioactivity retained on the filters was measured in PPO-POPOP-toluene scintillation liquid on Isocap 300, Nuclear Chicago.

peptidyltransferase. In our experiment peptidyltransferase activity was retained, so we can say that the inhibiting substance does not affect this enzyme.

On the other hand, cycloheximide, a specific inhibitor of translocation, added similarly to the incubation mixture, did not enhance the inhibitory effect of *Melissa*. We therefore suggest that the substance from *M. officinalis* inhibited the translocation step in peptide chain elongation.

To find the target site of this inhibitory effect, an experiment involving the formation of an [E-S] complex, EF-2 and ribosomes, was carrried out. The results (Fig. 4) show convincingly that the inhibitor from *M. officinalis* blocked the binding of EF-2 to ribosomes; the translocation step was inhibited and the peptide elongation terminated. We therefore conclude that EF-2 is a target site of the inhibiting substance from *Melissa*.

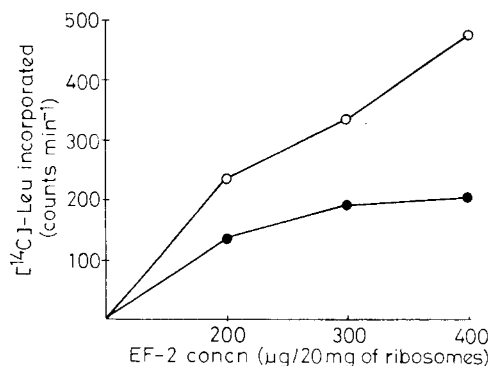

Fig. 4. The effect of the inhibiting substance from *Melissa* on the EF-2 binding with ribosomes. The y-axis is [^{14}C]leucine incorporated into proteins. ○——○ without inhibitor. ●——● with inhibitor. The binding of EF-2 with ribosomes was carried out in a mixture containing: ca 20 mg of ribosomes; increasing amounts of purified EF-2; 75 mM Tris HCl, pH 8·0; 7·5 mM $MgCl_2$; 100 mM NH_4Cl; 0·02 mM DTT; 0·25 mM GTP and 0·8 ml inhibitor fraction, equivalent to 32 mg of *Melissa* dry leaves. The mixture was incubated at 37 °C for 5 min, then cooled to 4 °C and overlayered to the top of a sucrose gradient (3·5 ml of 1 M and 3·5 ml of 0·5 M sucrose in buffered solution) and ultracentrifuged at 105 000g for 4 h. The sedimented ribosomes were washed and resuspended in 1 ml of buffered solution (0·35 M sucrose, 0·5 M Tris HCl, pH 7·4, 0·004 M $MgCl_2$). 0·02 ml of this solution containing ca 400 μg of ribosomes was added to the incubation mixture and [^{14}C]leucine incorporation into proteins was assayed (see Methods) in the presence of ca 100 μg of EF-1, but absence of EF-2.

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