Vitis vinifera as a Medicinal Plant

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Abstract

Grapes are the most important dietary sources of the main stilbenic phytoalexins (e.g. *trans*-resveratrol,) whose beneficial effects can be divided approximately into two main groups: protective/chemopreventive and killing/inhibiting effects. Using TLC, OPLC and HPLC as separation techniques, as well as MALDI MS data, we identified formaldehyde, in dimedone adduct form, and stilbene derivatives in different parts of the berries. For the biological evaluation of the fractions and reaction products various healthy (endothelial) and tumour cell lines (e.g. HT-29 human colon carcinoma), as well as *Botrytis cynerea* culture were used. *Trans*-resveratrol and related compounds have a characteristic á,â-double bond and phenolic hydroxyl groups, which determine their reactivity with other molecules. Such an interaction may be between *trans*-resveratrol and endogenous formaldehyde (HCHO) from labile bonds in animal and human tissues. The mobilization of HCHO with *trans*-resveratrol (first step) may cause e.g. a cardio protective or anticarcinogenic (e.g. initiation stage) effect and the reaction products (labile hydroxymethyl derivatives) (second step) may exert a killing (and/or apoptotic) activity to cancer cells and/or pathogens.

INTRODUCTION

Resveratrol, 3,5,4'-trihydroxystilbene, occurs mainly as the *trans* isomer in grapes (Langcake and Pryce, 1976) and grape products (Siemann et al., 1992) as well as in other plant species e.g. *Arachis hypogea* (Ingham, 1976); *Polygonum cuspidatum* (Arichi et al., 1982); and *Yucca schidigera* (Uenobe et al., 1997). The phytoalexin resveratrol, as a product of stilbene-synthase, has a function in plant responses to environmental stress and pathogens. A low incidence of coronary heart diseases may coexist with intake of a high fat diet, a phenomenon known as the French paradox (Renaud and de Lorgeril, 1992; Frankel et al., 1993). It has been suggested that red wine ingredients, including polyphenolic components, such as resveratrol, may contribute to this unusual phenomenon (Fremont et al., 1999). The up-to-date beneficial effects of *trans*-resveratrol can be divided approximately into two main groups: protective and killing effects. The main protective effects are as follows: cardio-protection (e.g. Frankel et al., l993; Kerry and Abbey, 1997), anti-oxidative effect (Fremont et al., 1999), estrogen antagonist (Turner et al., 1999), anti-platelet activity (Bertelli et al., l995), anti-mutagenic effect (Uenobe et al., 1997), anti-allergic action (Cheong et al., 1999), relaxant effect (Jager et al., l999) neuroprotective effect (Virgili and Contestabile, 2000), and decrease of hyperalgesia (Gentilli et al., 2001). Among the beneficial effects of *trans*-resveratrol, its killing-inhibiting activities are especially attractive. *Trans*-resveratrol is well-known as an antifungal constituent (Langcake and Pryce, 1976; Hain et al., 1993). Jang et al. (1998) reported more recently, using model assay systems, that *trans*-resveratrol acts as a pleiotropic biological effector to regulate the three major stages of chemical

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carcinogenesis as initiation, promotion and progression that underlie malignant transformation. More recently, it has been observed that *trans*-resveratrol selectively inhibits leukemia cells (Gautam et al., 2000). However, there are also some contrasting results in this diversity of activities of *trans*-resveratrol (e.g. Turrens et al., 1997; Mac Carrone et al., 1999). However, we do not know the mechanism of the activities of *trans*-resveratrol, with special emphasis on the two main groups of activity, the protective and killing-inhibiting effects. *Trans*-resveratrol exerts an antioxidant effect (e.g. Frankel et al., 1993). However, this does not give a response e.g. for the selective anti-leukaemic effect of *trans*-resveratrol (Gautam et al., 2000) or its beneficial effect in the three main stages of chemical carcinogenesis (Jang et al., 1998). *Trans*-resveratrol has a characteristic á,â-double bond and phenolic hydroxyl groups which determine its reactivity with other molecules. The parallel accumulation of *trans*-resveratrol and formaldehyde (HCHO) in parts of the berries (Király-Véghely et al., 1998), the dose-dependent activity of *trans*-resveratrol (Szende et al., 1998), as well as our previous observations gave us an impetus for studying the interaction between *trans*-resveratrol and HCHO in model reactions and in parts of the berries. It has became increasingly evident that there is a HCHO cycle in biological systems (Tyihák et al., 1998a) in which formation of the methyl group of L-methionine takes place through HCHO from S-adenosyl-L-methionine (SAM), which is linked to different enzymatic transmethylation reactions (Huszti and Tyihák, 1986). This paper describes the identification of *trans*-resveratrol and HCHO, in dimedone adduct form, in different parts of the berries of different grapevine cultivars and our preliminary observations on interaction between *trans*-resveratrol and HCHO in model reactions.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Merck Chemical Co. (Darmstadt, Germany) and Sigma Co. (St. Louis, USA) as well as REANAL Chemical Co. (Budapest, Hungary). Formaldemethone was made by a preparative method (Spencer and Henshall, 1955).

Quantification of Resveratrol and its Derivatives by HPLC

The HPLC equipment consisted of a Gyncotek (Germering, Germany) model M480 pump fitted with a Rheodyne (California, USA) model 8125 injector with a 20 µl sample loop, connected to a column (130x4.6 mm i.d.) filled with Chromspher C18 (5 µl of particle size) (Chrompack, Bergen op Zoom, The Netherlands). The UV detector employed was a Tosoh (Kyoto, Japan), model TSK 6040 UV instrument measuring at γ= 312 nm and the output was passed through an Electroflex GM (Szeged, Hungary) model EF 2012 ADDA converter and processed using a personal computer. The mobile phase was methanol: 0.01 N hydrochloric acid (77:23, V/V; pH 2.60) (at a flow rate of 1mL/min)

Identification of Resveratrol and its HCHO Reaction Products by Matrix Assisted Laser Desorption-Ionization Mass Spectrometry (MALDI-MS)

Either the authentic or isolated component in solution was mixed with 0.5 µL á-cyano-4-hydroxycinnamic acid (ACH) matrix [10 mg/mL ACH in acetonitrile: water (7:3, V/V)] directly on a disposable sample slide. The droplet was allowed to dry naturally prior to MALDI MS analysis. The sample preparation for the authentic substances was the same as before but using a standard solution (2x10-5 M) instead of diluted supernatant. The mass spectrometer used in this work was a Finnigan LASERMAT 2000 (Finnigan MAT Ltd., Hemel Hempstead, UK) (Whittal and Li, 1998).

Preparation of Reaction Mixtures between *trans*-resveratrol and HCHO and Analysis of Reaction Products by TLC and OPLC

Aliquot parts of diluted formalin and resveratrol in methanol were mixed at room temperature. The interactions between the two compounds were carried out at constant

and increasing concentrations of both resveratrol and HCHO. For the separation of reaction products from the interaction, a conventional TLC technique was used: sorbent layer, silica gel 60_{F254}; eluent, chloroform-methanol mixtures; spots or bands were evaluated visually by UV illumination (254 and 366 nm) and instrumentally by using a Shimadzu CS-930 densitometer (305 nm for resveratrol and oligomers; 295 nm for hydroxy-, hydroxymethyl derivatives).

Biological Investigations of *trans*-resveratrol. Antimitotic and Antiapoptotic Investigations

HT-29 human colon carcinoma (ATCC HTB-38) cells were cultured in 6 well and 24 well Grainer plates using RPMI supplemented with 10% fetal calf serum (Protein Biochemical Ltd., Gödöllő, Hungary), in a humidified CO_2 incubator. The number of cells at plating was 10^5/ml. Triplicate samples of cultured cells were treated with 1.0-10.0-100.0 μg/ml *trans*-resveratrol (Sigma), 24 h after plating. Samples were taken for cell count and for counting apoptosis and mitosis 24 and 48 h after *trans*-resveratrol treatment.

Bioautographic investigations. Separation of *trans*-resveratrol on TLC chromatoplate by automatic OPLC instrument (Mincsovics et al., 1996): P_{ext}, 50 bar, eluent, chloroform-acetonitril (40:60, V/V), R_{el}, 400 μl, V_{el}, 4100 μl. *Trans*-resveratrol, 2,4,8 μg (from left to right). Rf = 0.75.Biological evaluation: inoculation with *Botrytis cinerea* (necrotrophic microbe) or *Uromyces phaseoli* (biotrophic microbe). Incubation temperature: 21-37^{o}C and incubation time: 2-16 h. For visualization a tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)] (Sigma) was used.

RESULTS AND DISCUSSION

Identification and Measurement of Formaldehyde, as Formaldemethone, and *trans*-Resveratrol in Different parts of Berries and in Wines

The identification of HCHO in dimedone adduct form (m/z value of 293) and of *trans*-resveratrol (m/z value of 227) in an extract of the skin of blue grapes (Soproni Kékfankos variety) was carried out by MALDI MS. The amount of HCHO in dimedone adduct form (formaldemethone), and of resveratrol in parts of the berries of different grapevine varieties is given in Table 1. It can be seen from the data that there is a parallel accumulation of HCHO and *trans*- resveratrol in the different parts of the berries (Király-Véghely et al., l998). It has to be noted that the level of *trans*-resveratrol in the parts of white grape berries was always smaller than in that of blue grape berries. It was observable that the level of resveratrol is generally higher in red wines than in white ones (Table 2). These results are in agreement with the results for grape berries.

Interaction between *trans*-Resveratrol and HCHO in Formalin Solution and Analysis of Reaction Products

Trans-resveratrol is a well known common constituent of the human diet and it generates many diverse beneficial effects. We supposed that *trans*-resveratrol, as a special stilbene derivative with a special ¦Á,¦Â-double bond and 3 phenolic hydroxyl groups, could react with HCHO, that is, it is a natural HCHO capture molecule (Tyihák et al., 1998b). Table 3 shows the R_f values of the reaction products between *trans*-resveratrol and HCHO on the TLC chromatogram. It can be seen that in the original reaction mixture, under an UV lamp at 366 nm, yellow (Y), orange (O) and blue (B) bands are visible and at the origin there are macromolecule(s) (?), as well. Resveratrol itself (?) (B) or its modified forms give a blue fluorescence together with 2 polymer (PI and PII) derivatives, that is, the characteristic á,â-double bond is yet present. The fluorescence of Y and O bands decreases continuously and in about 4 hours totally disappears while the resveratrol band (B) and the two bands (PI and PII) under B show further the characteristic fluorescence. The phenolic hydroxyl groups of the yellow (Y) and orange (O) bands can be detected by Folin- Ciocalteu reagent.

Fig. 1 illustrates the MALDI MS spectrum of the fresh reaction mixture between *trans*-resveratrol and HCHO.

Main Results of the Biological Investigations

Trans-resveratrol influences dose dependently the proliferative and apoptotic activity of human tumour cells. Low doses (0.1-1.0 μg/ml) of *trans*-resveratrol enhance cell proliferation, while higher doses (10.0-100.0 μg/ml) induce apoptosis and decrease mitotic activity. Using a complex bioautographic system (BioArena), we could illustrate the antimicrobial activity of *trans*-resveratrol to biotrophic and necrotrophic microbes.

CONCLUSION

HCHO, as its dimedone adduct, was isolated and identified from different microbial, plant and animal tissues (Tyihák et al., 1998b) and now we have also identified it, in dimedone adduct form, from different parts of grape berries. HCHO is bound in cells to acceptor molecules (e.g. the guanidine group of free and bound L-arginine) with different binding forces (Trézl et al., 1981; Huszti and Tyihák, 1986; Heck et al., 1990). HCHO is practically under controlled regulation in the HCHO cycle (Tyihák et al., 1998a). However, the uncontrolled enzymatic production of HCHO from endogenous and/or exogenous substrates may exert a risk in pathogenesis of different disorders (Yu, 1997). It is also important here to stress that HCHO is not a side product but a basic and indispensable substance in hydroxymethyl groups required for various biological processes. According to our present observations *trans*-resveratrol is a natural, concentration-dependent HCHO capture molecule. It would seem that elimination of the uncontrolled HCHO molecules with this stilbene derivative may exert a double effect. The elimination of HCHO with resveratrol (first step) may have a cardio protective effect, be anticarcinogenic in the initiation stage, and have an antimutagenic effect; the reaction products between *trans*-resveratrol and endogenous HCHO (second step) may also act as killing/inhibiting factors against malignant cells and pathogens. These results add new dimensions to *trans*-resveratrol and mainly for endogenous HCHO. The HCHO mobilization from hydroxymethyl groups and subsequent reactivity process of the reaction products may be in general the basis of the diverse beneficial activities of *trans*-resveratrol. HCHO is a single, normal and indispensable component of all biological systems, while there are a lot of polyphenols and similar HCHO mobilization factors which can potentially mobilize HCHO and then normalize different abnormal processes. Therefore, these results give an impetus to the search for similar compounds in the biological world, mainly in the plant kingdom. There is also a possibility for the formation of such derivatives in the interaction between HCHO and *trans*-resveratrol in which the hydroxymethyl groups are near to the phenolic hydroxyl groups. It follows from these results that the common grape berries are one of the most important medicinal plants.

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Tables

Table 1. Distribution of HCHO (measuring in dimedone adduct form) and *trans*-resveratrol in the parts of berries of different grapevine cultivars

Grapevine cultivar fresh tissue	**HCHO, µg/g**	**Resveratrol, µg/g**
Chardonnay (white cultivar) sample		
Skin	6.0	1.40
Stem	6.3	0.50
Seed	1.7	0.14
Pulp	2.0	0.005
Soproni Kékfrankos (blue cultivar) sample		
Skin	2.8	10.00
Stem	4.3	7.00
Seed	0.9	3.00
Pulp	1.9	1.55

Table 2. Content of resveratrol in some Hungarian white and red wine samples

Wines	**Resveratrol, mg/l**
White wines	
Chardonnay	0.04
Hárslevelű	0.07
Szamorodni	0.05
Red wines	
Cabernet savignon	3.07
Kadarka	1.63
Egri bikavér	4.40

Table 3. R_f.x100 values of reaction products between trans-resveratrol and HCHO

Substances at 366 nm	**R_f.x100**
Yellow (Y) derivative	82
Orange (O) derivative	75
Blue (B) (*trans*-resveratrol (?))	66
Polymer (PI)	51
Polymer (PII)	40

Figures

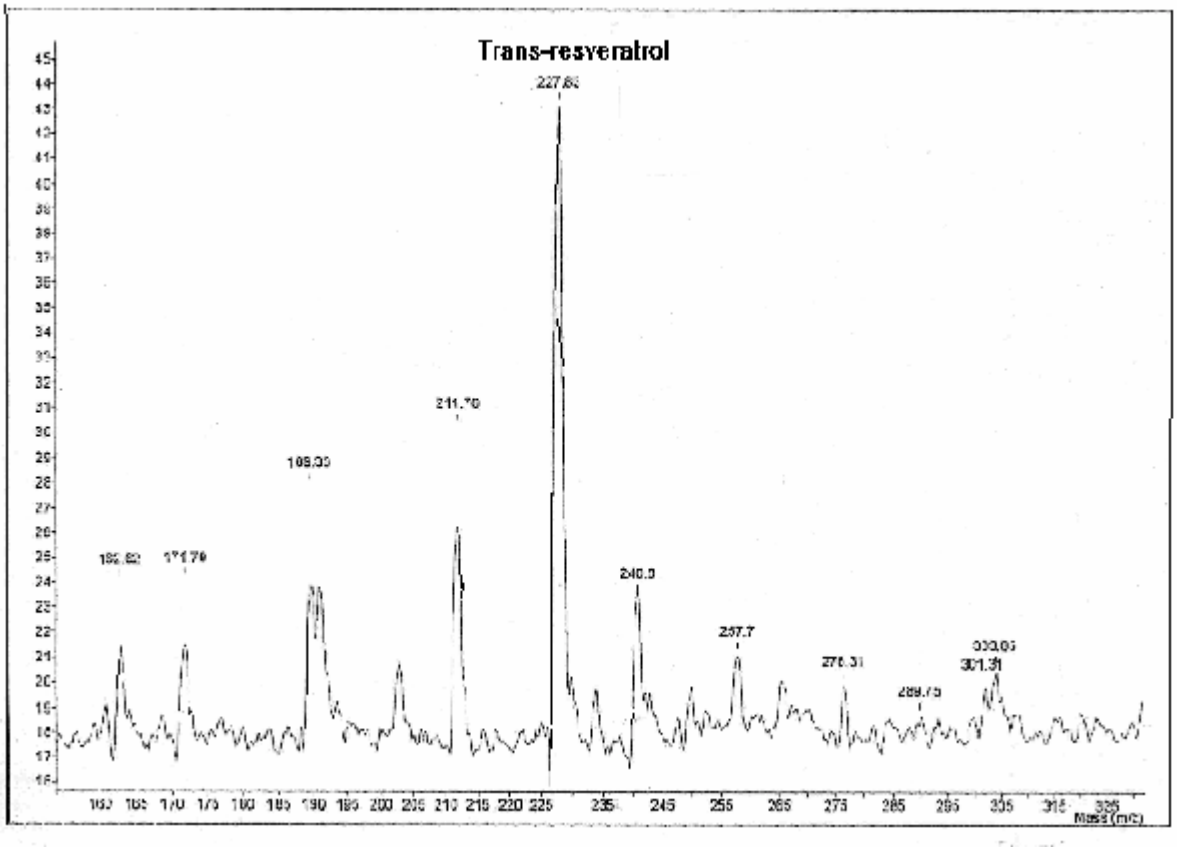

Fig. 1. MALDI MS spectrum of reaction mixture between *trans*-resveratrol and HCHO (see Materials and Methods)