Effects of Cyclodextrin Complexation on the Anti-cancer Effects of *Cotinus coggygria* Extract and its Constituents, Butein and Sulfuretin

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The importance of polyphenolic constituents and various plant extracts as anti-cancer products is well recognized. Their low bioavailability is however a major drawback in the efficient medicinal utilization. Cyclodextrin inclusion is a cost-effective approach to enhance drug delivery and stability. The present research reports the preparation and characterization of novel cyclodextrin complexes between a diethyl ether-soluble fraction of a Cotinus coggygria extract, and two hydrophilic β-cyclodextrin types. Subsequently, the growth inhibitory activities of the extract, two representative constituents (sulfuretin and butein), and their two respective cyclodextrin complexes was assessed against four cell lines: HeLa, A2780, MCF7 and MDA-MB-231. The extract displayed a valuable activity on ovarian carcinoma cells, inhibiting their growth in the low micromolar range. Butein displayed the highest activity against HeLa cervix carcinoma cell lines, inhibiting the growth of 82% of cells at 10μM (as a randomly methylated-β-cyclodextrin complex and in free form). Sulfuretin and its complexes had a generally lower cytotoxic activity. These are first results on the cytotoxic activity of cyclodextrin-complexed natural products from the European smoke tree (C. coggygria).

Keyword: plant extract, cyclodextrin complexation, sulfuretin, butein, cancer cell line, growth inhibition

Natural products are of particular relevance in the fight against cancer, and over 60% of drugs used in anticancer therapies are of natural origin [1]. In the last decades, a plethora of plant metabolites have been isolated displaying cytotoxic activities. Polyphenols are a recognized source of chemopreventive and anticancer agents. First evidence was gathered form epidemiological studies [2]. More elaborate investigation of their mechanism of actions were performed for the flavonol quercetin [3], the stilbene resveratrol [4], the flavanol ester epigallocatechin gallate, and curcumin [5]. Polyphenols modulate the transcription of genes, favour DNA repair, enhance apoptosis, inhibit angiogensis and reduce migration [6-8]. Young fustic (*Cotinus coggygria* Scop., Anacardiaceae) extract obtained from the heartwood contains a unique mixture of polyphenols: phenolic acids (gallic acid and its methyl ester), flavonols (catechin), proanthocyanidins (fisetinidol-(4α→8)-(+)-catechin and epifisetinidol-(4β→8)-(+)-catechin), flavanonols (fustin, dihydroquercetagetin), flavanones (butin, eriodictyol), flavonols (fisetin, quercetin), chalcones (butein), aurones (sulfuretin), beside a variety of flavonoid glycosides and dimers [9]. The plant has been used in ethnomedicine to treat skin conditions, wounds, gastritis, liver diseases and fever. Given the well-established properties of flavonoids to fight cancer, the aim of the present study was to explore on various cancer cell lines the effect of a *Cotinus coggygria* extract and of two marker compounds, sulfuretin and butein.

The main limitation of the efficient use of most phytocompounds is their low bioavailability [8]. Both sulfuretin and butein are compounds with a very low water solubility, and accumulate in the diethyl ether extracts of young fustic heartwood. Their isolation from *C. coggygria* extract was previously described [10]. The inclusion in cyclodextrins represents a cost-effective strategy for the efficient delivery of natural products, offering increased bioavailability, controlled release and protection against physico-chemical modifications [11]. Cyclodextrins are toroid-shaped oligosaccharides, able to entrap hydrophobic drugs and enhance their water solubility. Their use as pharmaceutical excipients is widely recognized, and cyclodextrin complexes have been shown to display anticancer activities [12,13]. Complexes of sulfuretin and butein with two cyclodextrin types: hydroxypropyl-β-CD (HPBCD) and randomly methylated-β-CD (RAMEB) were already obtained and characterized by our workgroup [10]. The present study reports the preparation and physico-chemical characterization of supramolecular complexes between *C. coggygria* diethyl-ether soluble extract and the two cyclodextrin types mentioned above. Moreover, the growth inhibitory activities of the extract, sulfuretin, butein and their two respective cyclodextrin complexes were tested against four cell lines: epitheloid cervix carcinoma (HeLa), ovarian carcinoma (A2780), and human breast adenocarcinoma (MCF7, MDA-MB-231).

Experimental part

Natural products

Branches and thick stems of young fustic plants from Caras-Severin county (South-Western Romania) were collected after positive identification at the Department of Pharmaceutical Botany; a voucher specimen is deposited at the Herbarium of the Faculty of Pharmacy, *Victor Babes* University of Medicine and Pharmacy of Timisoara. After removal of the outer bark from stems with a diameter of 7-

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10cm, heartwood was milled and used for extraction with methanol. The aqueous suspension of the methanol extract was further partitioned between petroleum ether, diethyl ether, ethyl acetate and *n*-buthanol. The diethyl ether fraction (code DEE) was further employed for the isolation of sulfuretin and butein. Detailed procedures are provided in our previous work [10].

The sulfuretin and butein content of the DEE extract was assessed by HPLC-DAD according to a method developed and validated earlier [14]. The relevant concentrations were 8.54% for sulfuretin, and 2.28% for butein.

Preparation of cyclodextrin complexes was performed by the kneading method, using a 1:2 mass ratio (extract: cyclodextrin). Cyclodextrins (HPBCD, RAMEB) were purchased from Cyclolab (Hungary). A mixture of extract and CD was triturated in a mortar, with the concomitant addition of a 50% ethanol solution in drops [23]. Kneading of the obtained paste was performed until the evaporation of the solvent. The mixture was then dried at room temperature for one day. The last steps were heating of the mixture at 105°C for several hours in an oven, pulverization, and sieving (using a 100μm sieve).

Differential Scanning Calorimetry (DSC)

The analysis was carried out with a Mettler-Toledo DSC1 instrument (Mettler-Toledo, Switzerland). Small portions of samples (4.1-4.3 mg) were placed in standard aluminum crucibles (40μL) with pierced caps. Heating was performed between 40-280°C in an inert Ar atmosphere at a heating speed of 5 degrees per minute. Empty aluminum crucibles with pierced caps were used as reference.

Particle size measurement

The size and the stability of particles were evaluated using ethanol solutions (1%, w/v) and a Cordouan Zetasizer instrument (Cordouan Technol., France) consisting of a Vasco Particle Size Analyzer and a Wallis Zetapotential Analyzer. Vasco Particle Size Analyzer parameters were set at: sample volume (~50 μL), temperature (25°C), time interval (6 μs), number of channels (450), laser power (100%), DTC position: UP, acquisition mode (continuous), and algorithms (Pade-Laplace, Cumulants). Wallis Zetapotential Analyzer parameters: sample volume (~1.2 mL), samples' *p*H at 25°C (~7.1), cuvette type (plastic, wavelength 380-780 nm), temperature (25°C), laser power (45%), applied field (automatic), resolution (medium, 0.8 Hz), 3 measures/sequence, and Henry function (Huckel).

Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Spectra were obtained in attenuated total reflectance (ATR) mode on a Perkin Elmer SPECTRUM 100 device. Spectra were collected in the 4000-600 cm^{-1} spectral range, with a resolution of 4 cm^{-1} and with 64 acquisitions as co-added scans. The samples were analyzed without further preparation.

Growth inhibition assay

Antiproliferative effects were assessed in vitro employing four human cell lines: HeLa (cervix adenocarcinoma), A2780 (ovarian carcinoma), MCF7 and MDA-MB-231 (breast adenocarcinoma) with the MTT ([3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide]) assay [15,16]. Cells were cultivated in culture medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic and antimycotic mixture (obtained from PAA Laboratories, Pasching, Austria). The growth environment was represented by a humidified atmosphere, containing 5% CO_2 and regulated at 37°C. Cancer cells (5000/well) were seeded onto a 96-well microplate and attached to the bottom of the well overnight. On the second day, 200 μL of new medium containing the test substances were added. After incubation for 72 h, the living cells were assayed by the addition of 20 ìL of 5 mg/mL MTT solution. The reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells were utilized as controls. All *in vitro* experiments were carried out on 2 independent microplates with at least 5 parallel wells. Stock solutions of the tested substances (1mM) and extract (1.65 mg/mL) were prepared with DMSO. The highest DMSO concentration (0.3%) of the medium did not have any significant effect on the cell proliferation. Final concentrations applied to the cells were 3 and 10 μM for the pure compounds, while for the extract the respective final concentrations were 5 and 16 μg/mL.

Results and discussions

Cyclodextrin complexes of plant extracts are less frequent than those of pure phytocompounds, however the literature provides several examples of successful encapsulation of *Panax ginseng* [17], *Ficaria verna* [18], *Euphorbia cyparissias* extracts [19], and propolis [20], in order to mask bitter taste or unpleasant smell and to enhance stability of active compounds. The successful encapsulation of diethyl-ether soluble extract of *C. coggygria* in two β-cyclodextrin types performed in the present work, could be demonstrated by thermal analysis, and infrared spectroscopy. Moreover, particle size was assessed, providing first physico-chemical data on these novel extract-CD entities.

With the aid of thermal analysis, qualitative and quantitative data about the physicochemical state of the *C. coggygria* extract trapped within the cyclodextrin cavity were obtained. In particular, disappearance or appearance of endothermic peaks and shifting of peaks to different temperature values were observed in order to point out possible changes in melting, boiling or sublimation points as well as modifications of the crystal lattice [21]. The thermograms of CD complexes with the DEE extract (fig. 1) indicate a slight dehydration of DEE-HPBCD occuring between 40 and 79°C with the maximum at 42°C. The thermogram of DEE-RAMEB (curve 2) exhibits a pronounced endothermic effect of melting process without loss of mass at 42°C, followed by a slight dehydration of DEE-RAMEB at a maximum of 62°C. The melting point of DEE was not observed inside the studied range of temperature (40-280°C), and its thermogram presents many artefacts above 235°C.

The successful entrapment of hydrophobic guests in the cyclodextrin cavity often produces shifting or disappearance of bands which are typical for the included product/molecule. On the other hand, the spectrum of a complexed molecule is expected to resemble with the one of the pure cyclodextrin. In the present case, the broad band in the range 3665-3000 cm^{-1} is due to the presence of H-bonded intermolecular O-H groups (peak at 3330 cm^{-1}). The lack of sharp bands in this spectral range suggests that no free O-H groups are present in this mixture, indicating a better interaction of the molecules in the solid-state. The C-H stretchings are observed as weak intense bands at 2919 and 2851 cm^{-1}. The solid-state interaction between C=O groups from the compounds lead to a combination band, intense at 1598 cm^{-1}, and a shoulder at 1672 cm^{-1}. Some characteristic bands for the 1,2,4-substitution mode of the benzene ring is sustained by the medium bands in the spectral ranges 796-838 and 965-

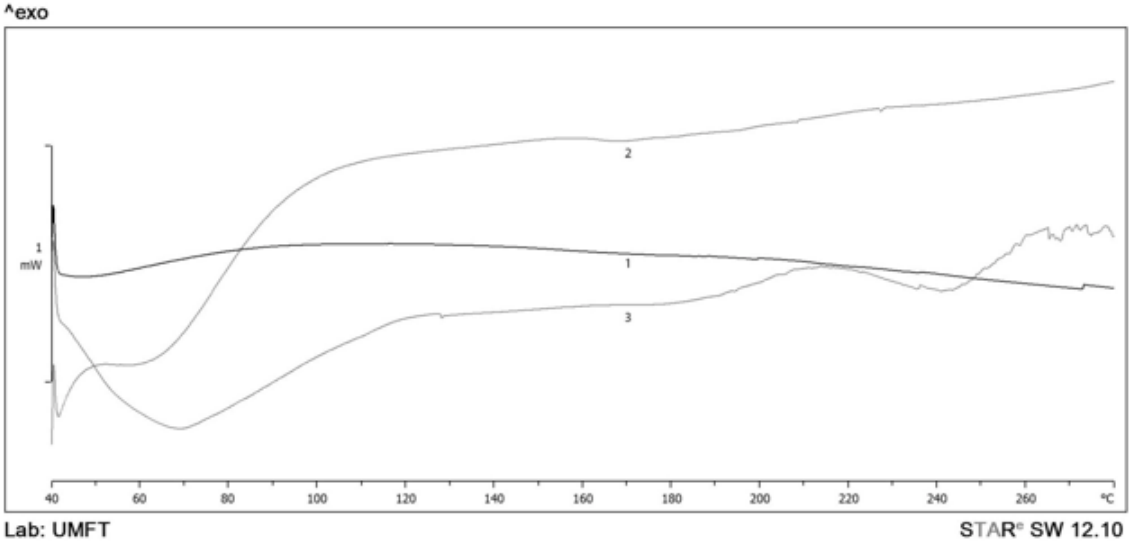


Fig. 1. Differential scanning calorimetry curves of dietyl ether-soluble extract (DEE) complexed with HPBCD (1), DEE-RAMEB (2) and free DEE (3)

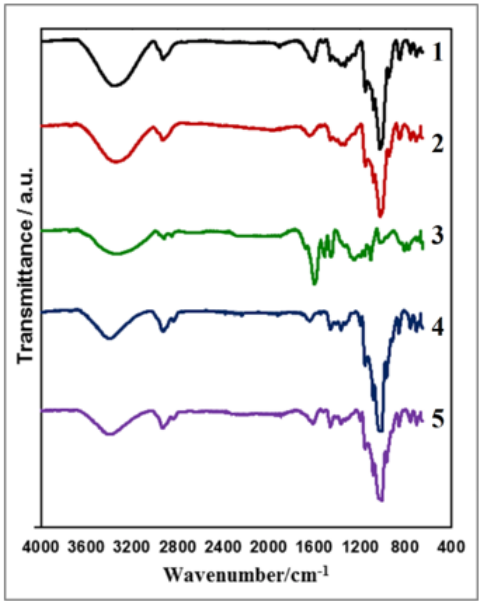


Fig. 2. ATR-FTIR spectra for DEE-HPBCD complex (1), HPBCD (2), free DEE (3), RAMEB (4), and DEE-RAMEB complex (5)

988 cm^{-1}, respectively. The benzofurane ring is represented by skeletal vibrations between 1349-1408 cm^{-1}.

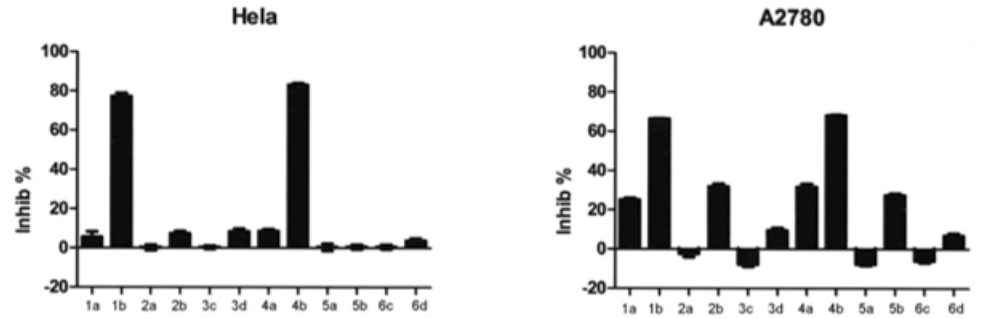
The measurement of size and stability of the obtained particles was performed with the Dynamic Light Scattering technique [22]. The particles consisting of *C. coggygria* extract complexed in HPBCD proved to be significantly smaller (table 1). As well, particles containing HPBCD are more stable, as shown by the higher zeta potential.

According to the literature, cyclodextrin complexation is included among the strategies to obtain nanosized particles that enhance the bioavailability, target delivery and stability of drugs [8]. It is a meaningful alternative to adapt the valuable therapeutic properties of phyto-compounds to the effective medical utilization. In general, nanosized particles are defined as having a size of 1-100nm, at which they display some unique properties and interactions. Conversely, larger particles with dimensions of up to 1000 nm are sometimes included in the group of nanomedicines, due to their favorable behavior and biological properties [23].

Following the successful preparation of cyclodextrin complexes hosting *C. coggygria* extract and marker compounds (sulfuretin, butein) [10] we proceeded to the analysis of their ability to inhibit the growth of cancer cells. Recent reports mention that the methanol extract of aerial parts from *C. coggygria* (leaves together with branches) has an anticancer effect against four human cells lines (A549, MCF7, TK6 and U937). The tested extract triggered coherent effects on the cell cycle and modified the expression of proteins involved in cell cycle regulation and apoptosis [24]. Departing from these favorable results, we investigated a standardized extract of the wood representing the diethyl ether soluble fraction of a methanol extract. At the employed concentration in the micromolar range (16µg/mL), the extract inhibited the growth of ovarian carcinoma cells by 50%, representing thus a valuable activity for a plant extract. The cytotoxic activity on the other three cell lines at the same applied concentration was lower, eliciting only 10-20% growth

Sample code	Particle size (nm)		Zeta Potential (mV)
	Mean ± SD	Polydispersity index	Mean ± SD
DEE-HPBCD	499 ± 13	0.4	18.32 ± 0.19
DEE-RAMEB	681 ± 20	0.3	12.51 ± 1.23
HPBCD	490 ± 12	0.1	18.96 ± 0.09
RAMEB	676 ± 16	0.2	12.64 ± 1.17

Table 1
THE ZETASIZER CHARACTERIZATION FOR SAMPLES



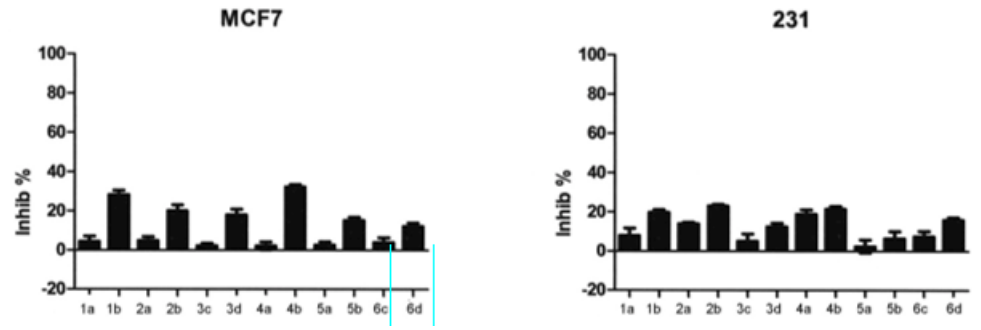
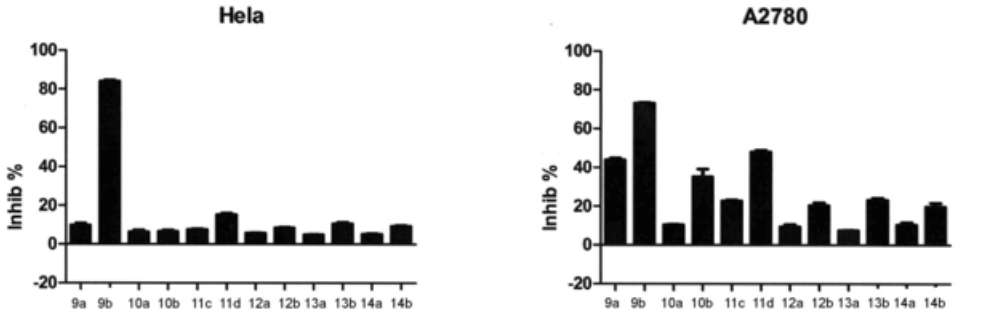


Fig. 3. Growth inhibiting activities of various cell lines, performed by natural products from *Cotinus coggygria* and their cyclodextrin inclusion complexes (part 1). Codes: 1-butein/HPBCD; 2-sulfuretin/HPBCD; 3-DEE extract/ HPBCD; 4-butein/RAMEB; 5-sulfuretin/ RAMEB; 6-DEE extract/ RAMEB; applied concentrations for pure compounds: 3μM (a) and 10 μM (b), applied concentrations for extract: 5μg/mL (c) and 16μg/mL (d).



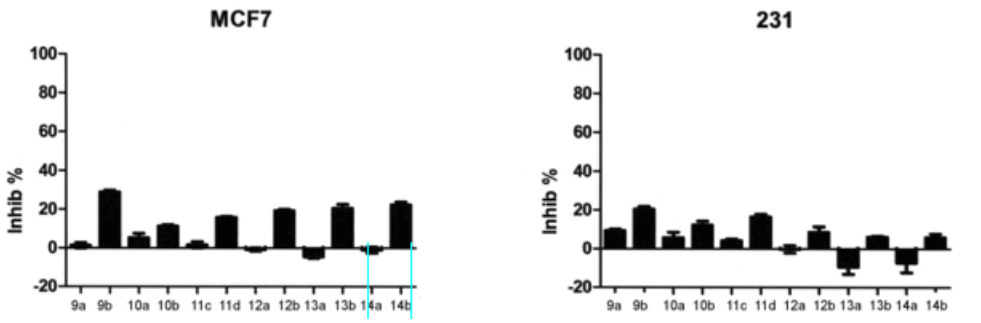


Fig. 4. Growth inhibiting activities of various cell lines, performed by natural products from *Cotinus coggygria* and their cyclodextrin inclusion complexes (part 2). Codes: 9-butein; 10-sulfuretin; 11-DEE extract; 12-HPBCD; 13-RAMEB; 14-DMSO (control); applied concentrations for pure compounds: 3μM (a) and 10 μM (b), applied concentrations for extract: 5μg/mL (c) and 16μg/mL (d).

inhibition. Among the four cell lines that were used, the cervix carcinoma cell lines proved to be the most sensitive to the effect of butein, which inhibited the growth of 82% of cells at 10μM (as a RAMEB complex and in free form), and 78% (as a HPBCD complex), respectively. The favorable anticancer activity of butein on various cell lines has as well pointed out by other researches, and underlying mechanism include induction of apoptosis and reversal of epithelial to mesenchymal transition [25]. On the other hand, sulfuretin was able to inhibit especially the human ovarian carcinoma cells (A2780), but its activity was generally lower than that of butein. In the employed experimental model, there were no significant differences in the inhibitory activity of free and complexed compounds. An IC_{50} value in the low micromolar range for butein is an encouraging value, warranting the research of this chalcone in free and complexed form as an organ-specific anti-cancer agent in animal models, especially to monitor the relevance of cyclodextrin inclusion *in vivo*.

Conclusions

The inclusion complexes of the diethyl ether soluble fraction of a methanol extract from C. coggygria wood, using two cyclodextrin types (hydroxypropyl-β-CD; HPBCD) and randomly methylated-β-CD; RAMEB) were succesfully obtained. Complex formation was substantiated by FTIR and thermal analysis by DSC with typical modifications of the spectra and thermograms, respectively. Particles obtained with HPBCD were smaller and more stable than those obtained with RAMEB. At the employed concentration in the micromolar range (16μg/mL), the extract displayed a valuable activity on ovarian carcinoma cells, inhibiting their growth by 50%. The cervix carcinoma cell lines proved to be the most sensitive to the effect of butein, which inhibited the growth of 82% of cells at 10μM (as a RAMEB complex and in free form). Sulfuretin had a generally lower cytotoxic activity, inhibiting especially the human ovarian carcinoma cells (A2780). Given the favorable effects of the extract and butein on cervix and

ovarian carcinoma cell lines as opposed to breast cancer cell lines, an organ-specific approach should be performed in animal models as a next step. The actual *in vivo* relevance of cyclodextrin inclusion complexes needs as well to be elucidated.

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