



ORIGINAL ARTICLE

# Synthesis, characterization of inclusion compounds of amygdalin with $\beta$ -cyclodextrin and sod-like activity and cytotoxicity on hela tumor cells



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**Abstract** In this work is presented the synthesis of the inclusion complexes of amygdalin with  $\beta$ -cyclodextrin by three methods: kneading, co-precipitation and freeze drying and their characterization.

The inclusion compounds were synthesized using a molar ratio of amygdalin:  $\beta$ -cyclodextrin of 1:1. Synthesized compounds were analyzed by FTIR spectroscopy, X-rays, thermal analysis and the results confirm the formation of inclusion compounds by amygdalin with  $\beta$ -cyclodextrin.

The studies carried out have shown the protective effect of amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze drying, kneading and coprecipitation against free radicals (SOD-like activity

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*in vivo*) and the results of *in vitro* cell cytotoxicity of the compounds on HeLa cell line compared to pure amygdalin. Compound obtained by freeze drying has the best SOD-like activity and cytotoxicity on HeLa tumor cells, so it may be considered as potential therapeutic agent.

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## 1. Introduction

Amygdalin (AMG) also known as B17 vitamin, or previously called laetrile represent a chemical compound from the largest class of nitrilosides. Such compounds which are found in the prunasian family seeds (seeds of apricots, almonds, peaches, apples, and other rosaceous plants) represent cyanide containing natural substances (Chang et al., 2006). Among the other prunasins, Armeniaceae seeds have been used for the treatment of asthma, bronchitis, emphysema, leprosy, colorectal cancer, leucoderma, and pain, throughout time (Hwang et al., 2003; Chang et al., 2005; Jung et al., 2019). Chemical structure of amygdalin consists from two molecules of glucose linked with a molecule of benzaldehyde, which is responsible for its analgesic action, and one of hydrocyanic acid, having an anti-neoplastic action. Apart from the above indications, amygdalin has been used to treat cancers and relieve pain (Ellison et al., 1978; Sim et al., 2000; Fukuda et al., 2003), D-amygdalin (D-mandelonitrile- $\beta$ -D-gentiobioside) is known to exhibit selective antinoplastic action (Koo et al., 2005).

AMG chemical formula is presented in Fig. 1.a, and the chemical name of amygdalin according to IUPAC is [(6-*O*- $\beta$ -D-glucopyranosyl- $\beta$ -D-glucopyranosyl)oxy](phenyl)acetonitrile.

As pure compound amygdalin is a slightly hygroscopic product which can be found as white or slightly yellow powder, or as slightly yellow crystals (Chang et al., 2006). From medical point of view, amygdalin's limited solubility in water, which decreases as temperature rises, represents the main issue. Amygdalin presents a good solubility in alcohol and methanol. A different approach used to increase amygdalin's solubility concomitant with the increase of its bio-availability can be represented by preparation of his complexes with cyclodextrins.

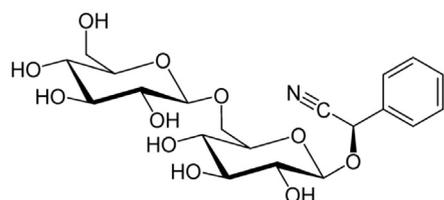
Cyclodextrins are a class of cyclic oligosaccharides which are prepared from enzymatic starch hydrolysis. The most known and used cyclodextrins consists in 6, 7 or 8 units of D-glucopyranose which are linked together by oxygen bridges. These compounds are designed as  $\alpha$ ,  $\beta$ , and  $\gamma$  cyclodextrin, which are also known as parental cyclodextrins, because derivative cyclodextrins compounds are obtained by attaching different functional groups at parental cyclodextrin structures. Pharmaceutical usage of cyclodextrins is possible mainly due

to their ability to form inclusion stable complexes with different active substances (substances responsible for therapeutic effect).  $\beta$ -cyclodextrin and its derivative as hydroxypropyl- $\beta$ -cyclodextrin and methyl- $\beta$ -cyclodextrin are compounds which present a high solubility in water. Due to the higher solubility in water correlated with the ability to form stable inclusion complexes with different active compounds, it can be used  $\beta$ -cyclodextrin to pharmaceutical preparation of new substances with significantly improved bio-availability of active compound along with reduction of different adverse effects. Experimental tests proved that the active compounds encapsulation in cyclodextrins cavity modify only physical properties (solubility, chemical reactivity, odor) having no effects over the therapeutic properties. In real practice the cyclodextrins were used for development and preparation of active compound with controlled release.

$\beta$ -cyclodextrin is the most studied in its class of compounds proving that it is a safety compound which can be easily metabolized by humans. Long term feeding studies proved that  $\beta$ -cyclodextrin have no toxicology significance when the diet included up to 5% of compound. Also, such tests proved that this cyclodextrin present no toxicity for subject's reproductive system. Main advantage of  $\beta$ -cyclodextrin usage for drugs encapsulation is represented by its high resistance at the action of salivary or pancreatic  $\alpha$ -amylases. Its ring is open in the colon by enzymes from *Bacteroides* species which lead at the formation of maltoheptaose fragments (Hedges 2009).

Recently different active compounds were used for inclusion of pharmaceutical substances with cyclodextrins: repaglinide (Nicolescu, Arama et al., 2010), \*\*sulphonamidic diuretics (Soica et al., 2006a, 2006b; Aluas et al., 2007; Soica et al., 2007; Şoica et al., 2008), pentacyclic triterpenes (Dehelean et al., 2008), fosinopryl natrium (Sbarcea et al., 2011), erythromycin (Marian et al., 2011).

AMG it is a natural cyanogen glycoside which can be split by beta-glucosidase producing free cyanide. In present study we aimed to activate amygdalin directly at the tumor site, so the malign cells can be killed without systemic toxicity. In order to achieve this aim the amygdalin molecule has been included into the cyclodextrin cavity and obtained inclusion complexes (Fig. 1b) were tested *in vitro* and *in vivo* to prove



a) Chemical formula of amygdalin



b) inclusion complexes preparation

Fig. 1 Chemical formula of amygdalin.

compounds specificity and cytotoxicity. Other aim of this work was to investigate the molecular encapsulation of amygdalin in the  $\beta$ -cyclodextrin cavity by using FTIR spectrometry, X-ray diffraction (XRD) and differential scanning calorimetry (DSC). In order to prove the molecular encapsulation of amygdalin was studied the protective effect of produced complex against free radicals produced by oxidative agents. Protective effects were evaluated by using a strain of *S. Cerevisiae* which is known for its ability to delete / insert the SOD1 gene.

## 2. Materials and methods

All materials were of reagent grade and were used without further purification. Amygdalin and  $\beta$ -cyclodextrin were purchased from Sigma Aldrich GmbH, Germany.

FTIR spectra were recorded using a JASCO 6100 FTIR spectrometer in the 4000–400  $\text{cm}^{-1}$  spectral domain with a resolution of 4  $\text{cm}^{-1}$  by using KBr pellet technique.

X-ray diffractograms were recorded using a Bruker D8 Advance diffractometer in the angular domain  $2\theta = 2\text{--}50^\circ$  using Cu  $K\alpha_1$  radiation. In order to increase the resolution a monochromator was used to eliminate the  $K\alpha_2$  radiation.

The DSC thermograms were obtained with a DSC-60 Shimadzu differential scanning calorimeter. The samples' heating was done with a rate of 20  $^\circ\text{C}/\text{min}$  in the 20–500  $^\circ\text{C}$  interval by using sealed Al cells, in nitrogen atmosphere.

### 2.1. *In vivo* SOD-like activity

The protective effect of pure AMG and of amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, coprecipitation and kneading against free radicals produced by oxidative agents had been determined.

The SOD-like activities of amygdalin- $\beta$ -cyclodextrin compounds were evaluated using a strain of *S. Cerevisiae*  $\Delta\text{sod1}$  (ATCC96687) (American Type Culture Collection), which has the ability to delete/insert the SOD<sub>1</sub> gene encoding the synthesis of Cu<sub>2</sub>Zn<sub>2</sub>SOD. Yeast strains lacking CuZnSOD ( $\Delta\text{sod1}$ ) were first created by deletion-replacement mutations in the *S. cerevisiae* CuZnSOD gene. Higher levels of oxidative damage to both cytosolic and mitochondrial proteins in the  $\Delta\text{sod1}$  strain provide evidence that CuZnSOD protects yeast from oxidative stress (Sheng et al., 2014). The characteristics of *S. Cerevisiae*  $\Delta\text{sod1}$  (ATCC96687) are: MAT aura 3–52 trp1-289 his3- $\Delta$ 1 leu 2–3 leu 2–112 sod1: URA3). The Cu<sub>2</sub>Zn<sub>2</sub>SOD is the main SOD in the cell and is localized in the cytoplasm.

Yeast cells were grown in YPD reach medium (1% yeast extract, 2% peptone and 2% glycerol). The used culture medium does not contain glucose, it contains glycerol instead, because in its presence the levures can breathe. This is determinant, as free radicals are going to be generated during the breath processes taking place in the mitochondria (Sas et al., 2007).

Solid media used for cell culture contained 1.5% agar. Cell density from cultures grown overnight was determined by cell counting in a “Nebauer” hematimetre.  $10^6$  cells were resuspended in 15 mL of melted solid YPD media kept at 45  $^\circ\text{C}$ . Solutions of pure AMG and amygdalin- $\beta$ -cyclodextrin compounds in a mixture of DMSO: EtOH (1:4) at increasing concentrations (30, 50, 70  $\mu\text{m}$ , see table 2) were added to the growth medium. Cell suspensions were poured into Petri dishes and allowed to solidify at room temperature. Paper

disks measuring 6 mm in diameter (Antibiotica test Blättchen) containing 5  $\mu\text{L}$  of a 5 mM menadione solution in ethanol or 5  $\mu\text{L}$  of 17.5% H<sub>2</sub>O<sub>2</sub> have been used. The diameters of clear zones around the disks, measured after 3 days of incubation at 28  $^\circ\text{C}$ , were taken as a quantitative estimate of the protective action (Marian et al., 2018b).

### 2.2. Cell culture

Two cell lines were used for determining the cytotoxicity of amygdalin- $\beta$ -cyclodextrin compounds, a human cervical carcinoma line (HeLa) (Sigma) and a normal fibroblastic epithelial cell line (HFL1) (Sigma). The cell lines were maintained in DMEM (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Hyclone), 1 mM glutamine (Sigma-Aldrich), 1% antimycotic antibiotic 100x (Sigma-Aldrich). The cells were cultured at 37  $^\circ\text{C}$  in an atmosphere of 5% CO<sub>2</sub> and 95% relative humidity (Niculescu et al., 2014; Hangan et al., 2016; Hangan et al., 2017).

### 2.3. Cytotoxicity assays

For the cell survival both cell lines were plated ( $1 \times 10^5$  cells/ well) in 96-well plates for 24 h in normal propagation media. The culture medium was then replaced with complete medium containing pure amygdalin and three type of amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation, in six different concentrations (100  $\mu\text{M}$ , 50  $\mu\text{M}$ , 25  $\mu\text{M}$ , 12.5  $\mu\text{M}$ , 2  $\mu\text{M}$  and 0.2  $\mu\text{M}$ ). The amygdalin- $\beta$ -cyclodextrin compounds were initially dissolved in DMSO, followed by serial dilutions in media until the final concentration of DMSO was less than 1%. The negative controls were represented by cells lines cultivated in normal expansion medium added with the same amount of diluted DMSO, while the positive control was represented by Cisplatin (Ebewe Pharma Ges.m.b.H. Nfg. KG, Austria) in the same concentration as the amygdalin- $\beta$ -cyclodextrin compounds. Each experiment was carried out in triplicate. The cellular viability was determined using the MTT assay at 24 and 48 h. The formazan particles were solubilized with dimethyl sulfoxide (DMSO) (Sigma). The absorbance was read at 550 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA) (Hangan et al., 2012; Boodram et al., 2016; Sevastre et al., 2017).

### 2.4. Statistics

All the experiments were conducted in triplicates and data are displayed as Mean  $\pm$  SD, representing the average of independent experiments performed in triplicate. The IC<sub>50</sub> values representing the amygdalin- $\beta$ -cyclodextrin compounds concentration required to inhibit 50% of cell proliferation were calculated from the dose response curve using non-linear regression. Statistical values were generated using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

## 3. Results and discussions

### 3.1. General procedure for the synthesis of the $\beta$ -cyclodextrin inclusion compound

*Kneading method* – were triturated together 0,0005 mol of  $\beta$ -cyclodextrin with 0,0005 mol of amygdalin (1:1 M ratio) in a

mortar by adding drops of ethanol (0.5 mL each). Obtained paste were triturated till the solvent's evaporation, the process was repeated several times, so that the total time period to be about 40 min.

**Coprecipitation method** – 0,0005 mol amygdalin is dissolved in 5 mL of ethanol, simultaneously 0,0005 mol  $\beta$ -cyclodextrin are dissolved in 5 mL distilled water (molar ratio 1:1), over cyclodextrin aqueous solution is added drop by drop amygdalin alcoholic solution under continuous stirring, the solvents are then evaporated at 50 °C in a drying stove to obtain the complex powder.

**Freeze-dryer** – Equimolar amounts of amygdalin and  $\beta$ -cyclodextrin were dissolved in water and mixed for 2 h at 35 °C wrapped in the aluminum foil to protect the solutions from the direct sun light. After the equilibration period of 24 h the clear solutions were frozen and subsequently lyophilized in a freeze-dryer of Alpha 1–2 LD type.

3.2. Physical – chemical characterization of synthesized compounds was realized by using FT-IR spectrometry, XRD diffraction, and thermal analysis.

### 3.2.1. FTIR spectrometry of amygdalin- $\beta$ -cyclodextrin inclusion compounds

In Figs. 2–4 are presented the IR spectra recorded for: amygdalin,  $\beta$ -cyclodextrin and prepared complexes of amygdalin with  $\beta$ -cyclodextrin.

By analyzing the FT-IR spectra recorded were observed the presence of some vibrations located at approximately  $3400\text{ cm}^{-1}$ , which are associated with the stretching vibration of  $\text{—OH}$  groups from unassociated alcohol molecules. Into the spectra recorded for  $\beta$ -cyclodextrin were observed some intense bands located between  $3400$  and  $302\text{ cm}^{-1}$ , bands which are related to the  $\text{—OH}$  groups stretching, and in region  $3000$ – $2900\text{ cm}^{-1}$  were located the stretching vibrations of  $\text{—CH}_2$  and  $\text{—CH}$  groups.

One important vibration which is represented by the stretching vibration of  $\text{C—O—C}$  group (etheric group), vibration which were presented into the amygdalin spectra (at  $1176\text{ cm}^{-1}$ ), cyclodextrin spectra (at  $1180\text{ cm}^{-1}$ ), and into

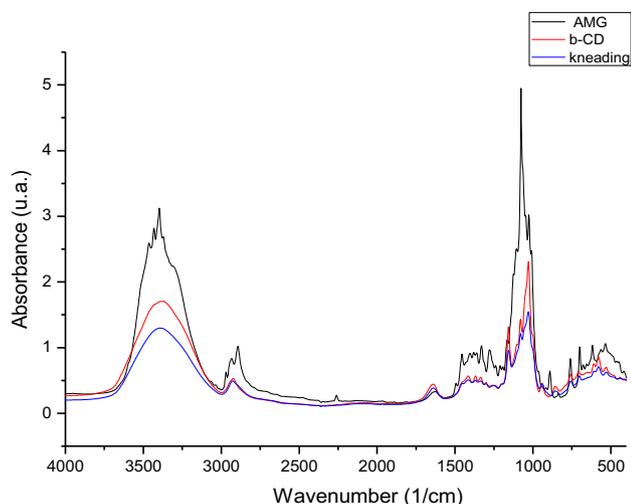


Fig. 2 FTIR spectra of the inclusion compound obtained by kneading procedure (*kn*) on  $4000$ – $400\text{ cm}^{-1}$  spectral domain.

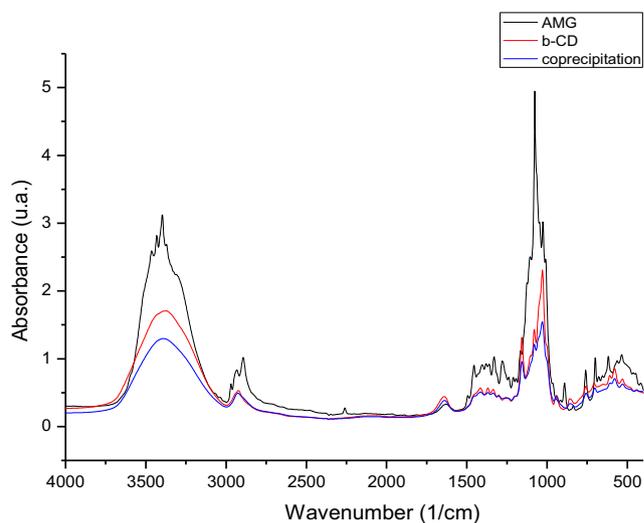


Fig. 3 FTIR spectra of the inclusion compound obtained by coprecipitation procedure (*co*) on  $4000$ – $400\text{ cm}^{-1}$  spectral domain.

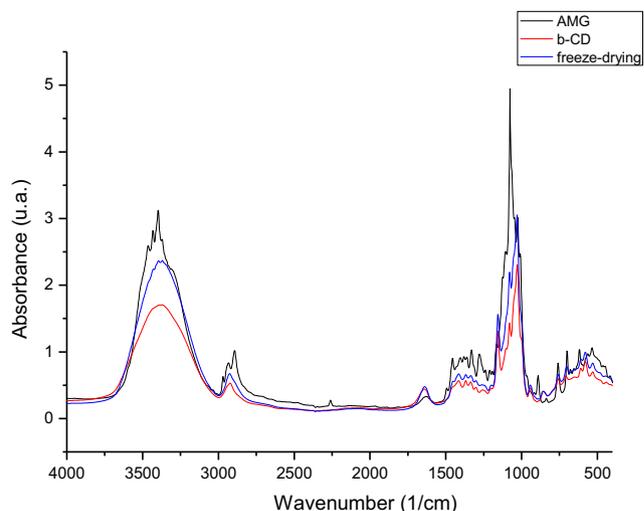


Fig. 4 FTIR spectra of the inclusion compound obtained by freeze-drying procedure (*fd*) on  $4000$ – $400\text{ cm}^{-1}$  spectral domain.

the spectra of prepared complexes (at  $1184$ ,  $1174$ , and  $1178\text{ cm}^{-1}$ ).

Into the spectra recorded for produced complexes were not identified the peaks located at  $3435$  and  $3404\text{ cm}^{-1}$ , peaks which are presented into the amygdalin spectra and which correspond to the stretching vibrations of  $\text{—OH}$  groups bonded by hydrogen bonds. Absence of these peaks can be related to the formation of complexes, by formation of new bonds between cyclodextrin hydroxyl groups and amygdalin hydroxyl groups.

Other vibration founded in all recorded spectra (amygdalin –  $885\text{ cm}^{-1}$ ,  $\beta$ -cyclodextrin  $860\text{ cm}^{-1}$ , and produced complexes –  $855$ ,  $853$ ,  $848\text{ cm}^{-1}$ ) is the stretching vibration of  $\text{—CH}$  bond from aromatic ring. Into the amygdalin spectra was identified the presence of the stretching vibration of nitrile group located at  $2259\text{ cm}^{-1}$ , vibration with a low intensity, which cannot be identified into the spectra recorded for

produced complexes. Other vibration related to the stretching of  $-\text{CH}$  bonds from aromatic ring were identified into the amygdalin spectra at  $699\text{ cm}^{-1}$ , also into the prepared complexes at  $688$ ,  $691$  and  $691\text{ cm}^{-1}$ .

One vibration with high intensity corresponding to the stretching vibration of  $\text{C}=\text{O}$  and  $\text{C}-\text{C}$  bonds is observed into the amygdalin spectra at  $1020\text{ cm}^{-1}$ , these vibrations being located also into the complexes recorded spectra at approximately same wavelength.

### 3.2.2. X Ray-diffraction of amygdalin- $\beta$ -cyclodextrin inclusion compounds

Analyzing the diffractograms depicted in Fig. 5 can observe that some of the peaks corresponding to the pure compounds are presented into the produced complexes, being shifted, and having lower intensities. Also were observed that in spectra recorded for produced inclusion complexes some peaks disappear, or present a diminished intensity. Such behavior demonstrates that into the produced compounds exist some interactions between the guest molecule (amygdalin) and the host one ( $\beta$ -cyclodextrin), confirming the formation of new compounds.

Analyzing the data presented in Fig. 5 can observe that into the patterns recorded for obtained complex the peaks of amygdalin are superposed with the peaks of  $\beta$ -cyclodextrin leading at an increase of height. The X-ray changes represent the proof of complexation between the guest molecule-amygdalin and the host molecule- $\beta$ -cyclodextrin.

### 3.2.3. Thermal analysis of amygdalin, $\beta$ -cyclodextrin, amygdalin- $\beta$ -cyclodextrin inclusion compounds

Analyzing the TG and DSC (Fig. 6) curves recorded for pure amygdalin were observed a small mass loss (around 1.59%) into the temperature range between  $50$  and  $160\text{ }^\circ\text{C}$ , with a maximum when the temperature has the value of  $119\text{ }^\circ\text{C}$ . This mass loss can

be associated with loss of some small quantity of water which was adsorbed from atmosphere during amygdalin manipulation. Into the temperature range  $200$ – $500\text{ }^\circ\text{C}$  can be observed the presence of two peaks, first located at  $226\text{ }^\circ\text{C}$  which correspond to the amygdalin melting, followed by his thermal decomposition. During this temperature interval the mass loss is 84.64%, both processes being endothermal one. At temperature of  $599.6\text{ }^\circ\text{C}$  the residual mass is 13.77% (Marian et al., 2018a).

From the TG spectra recorded for  $\beta$ -cyclodextrin (Fig. 6) can observe that the studied compound had a first mass loss between  $50$  and  $114.7\text{ }^\circ\text{C}$ , loss which is associated with water loss. During this process 13.59% from initial mass was lost. Into the temperature range  $114.7$  and  $306.5\text{ }^\circ\text{C}$   $\beta$ -cyclodextrin is stable, no mass loss being observed. Into the temperature interval  $306.5$  and  $377.2\text{ }^\circ\text{C}$  the thermal decomposition of  $\beta$ -cyclodextrin is taking place, when 72.22% from initial mass were lost. Both processes are endothermic one.

The thermal behavior of the produced complexes is similar. From the TG curves recorded for prepared complexes (Fig. 7) can observe that three different processes had been identified on the curves. Parameters associated with thermal behavior of prepared complexes are presented in Table 1.

After that was determined the solubility of pure AMG and of prepared inclusion complexes, obtaining for pure AMG a value close to the value from literature ( $0.09\text{ g mL}^{-1}$ ). For inclusion complexes have been obtained values which indicate an increase of the solubility: freeze drying  $0.19\text{ g mL}^{-1}$ ,  $0.17\text{ g mL}^{-1}$  for coprecipitation and  $0.12\text{ g mL}^{-1}$  for kneading. Obtained values indicate the preparation of desired inclusion complexes.

### 3.3. In vivo SOD-like activity

The *in vivo* SOD-like activity of pure AMG and of amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying,

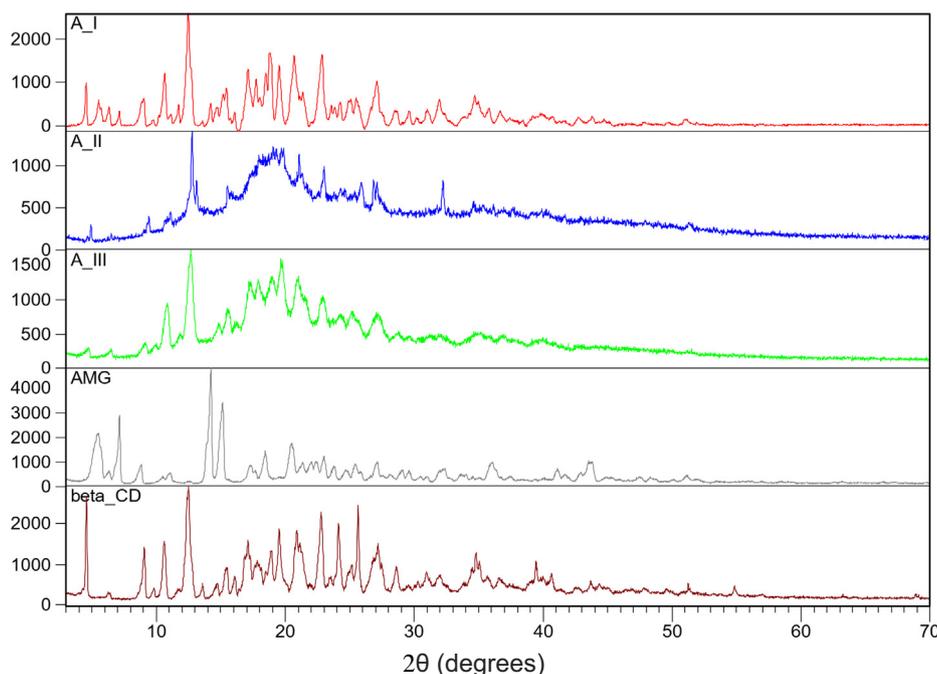


Fig. 5 X-ray diffraction patterns of  $\beta$ -cyclodextrin, amygdalin and of the inclusion compounds obtained by coprecipitation (AIII), kneading (AII) and freeze-drying (AI) procedures.

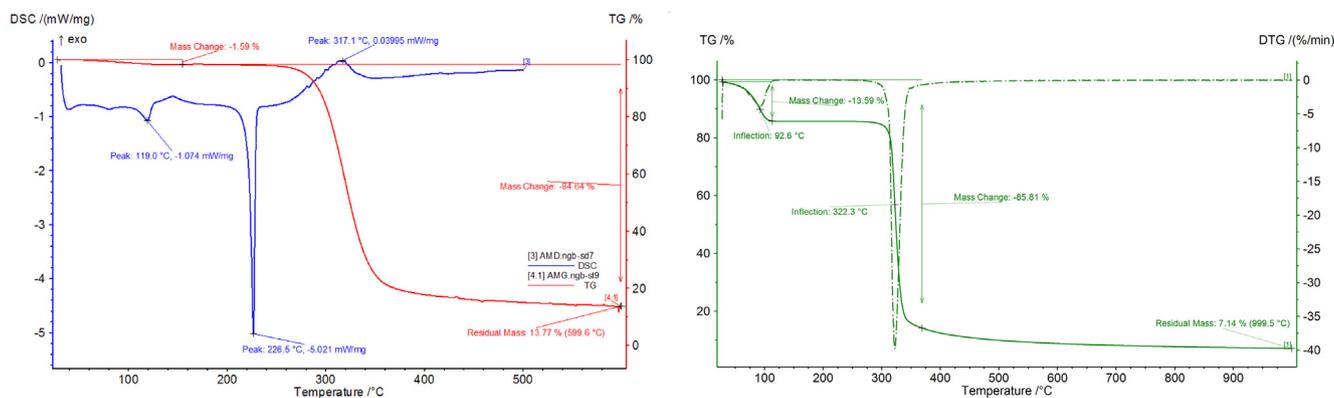


Fig. 6 TG and DSC curves of amygdalin and TG and DTG of  $\beta$ -cyclodextrin.

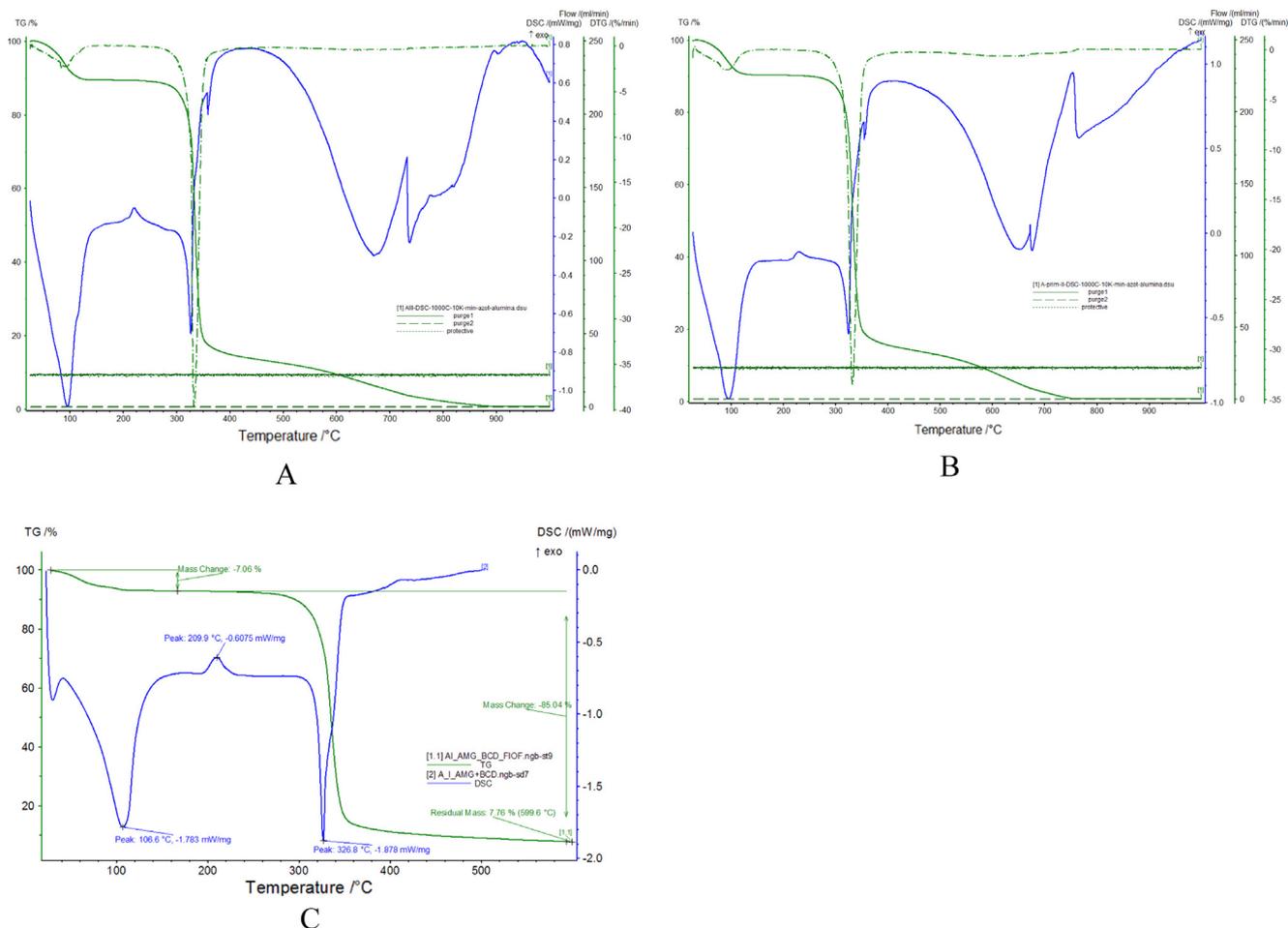


Fig. 7 TG, DTA and DSC curves of the inclusion compounds of amygdalin with  $\beta$ -cyclodextrin obtained by kneading (*kn*) procedures (A), coprecipitation (*co*) procedures (B) and freeze-drying (*fd*) procedures (C).

kneading and coprecipitation was quantified by a method based on the protection against free radicals provided by the extracts to the yeast *S. Cerevisiae* (Gonzalez-Alvarez et al., 2008; Marian et al., 2017). The SOD-mimetic activity of pure AMG and of amygdalin- $\beta$ -cyclodextrin compounds on cell growth with a  $\Delta$ sod1 mutant treated with menadione or  $H_2O_2$  had been evaluated. The oxidative stress is produced by two oxidative agents: menadione which toxicity is due to

the superoxide radicals' production and  $H_2O_2$  which toxicity is due to  $OH^\cdot$  radicals.

It will be considered that pure AMG and amygdalin- $\beta$ -cyclodextrin compounds have a SOD-like activity if a decrease of the diameter of the inhibition zone is registered versus the control zone. The efficacy will then be evaluated by comparison of the diameter of the inhibition area for pure AMG, amygdalin- $\beta$ -cyclodextrin compounds

**Table 1** Parameters associated with thermal behavior of prepared complexes.

Prepared complex	Thermal decomposition phase	Temperature interval (°C)	T <sub>pic</sub> DTG (°C)	Mass loss (%)	Process nature	Residual mass (%)
Kneading complex	I	50–120	90.0	10.00	Endothermic dehydration	1.12
	II	120–260	225.0	0.00	Exothermic melting	
	III	260–400	332.0	75.55	Endothermic decomposition	
	IV	400–700	680.0	13.33	Endothermic decomposition	
Coprecipitation complex	I	50–120	98.0	8.88	Endothermic dehydration	1.12
	II	120–260	225.0	0.00	Exothermic melting	
	III	260–420	334.4	65.64	Endothermic decomposition	
	IV	420–750	668.0	24.37	Endothermic decomposition	
Freeze-drying complex	I	50–150	106.6	7.06	Endothermic dehydration	7.76
	II	150–280	209.9	0.00	Exothermic melting	
	III	280–400	326.8	85.18	Endothermic decomposition	

obtained by freeze-drying, kneading, coprecipitation and control.

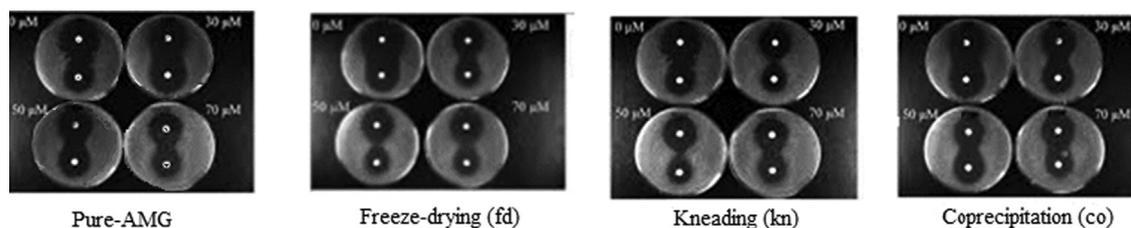
Fig. 8 shows the results for pure AMG and for amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation on the growth of the  $\Delta$ sod1 mutant against free radicals produces by H<sub>2</sub>O<sub>2</sub> and menadione. In the presence of amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation at 30, 50 and 70  $\mu$ M concentrations, a significant reduction of the inhibition area is observed when the oxidative stress is produced by both menadione and H<sub>2</sub>O<sub>2</sub>. In the presence of pure AMG a reduction of the inhibition area is also observed, but less pronounced than for all the amygdalin- $\beta$ -cyclodextrin. The diameter of the inhibition area for pure AMG for amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation in different concentrations, using menadione and H<sub>2</sub>O<sub>2</sub> are given in Table 2.

The reduction of the inhibition area is between 39 and 43% for the amygdalin- $\beta$ -cyclodextrin obtained by freeze-drying, 36–40% for the amygdalin- $\beta$ -cyclodextrin obtained by kneading and 25–28% for the amygdalin- $\beta$ -cyclodextrin obtained by coprecipitation, and 23–26% for pure AMG against oxidative

stress generated by menadione. The protective activities of pure AMG and of amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation do not seem to be dependent on amygdalin- $\beta$ -cyclodextrin compounds concentration.

The protection of the amygdalin- $\beta$ -cyclodextrin compounds against free radicals generated by H<sub>2</sub>O<sub>2</sub> is lower than in the case of free radicals generated by menadione. Menadione-induced superoxide dismutase very quickly to H<sub>2</sub>O<sub>2</sub> which can be easily detected in different systems. Thus, effect of menadione can be related to both superoxide and hydrogen peroxide (Timoshenko et al., 1996). The compound obtained by freeze-drying produce a reduction of the inhibition diameter about 28–32%, the compound obtained by kneading between 23 and 25%, while the compound obtained by coprecipitation only between 17 and 19%, while pure AMG only between 15 and 16%. Nor in this case the protective action produced by pure AMG and by amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation does not depend on amygdalin- $\beta$ -cyclodextrin compounds concentration.

In conclusion, all three amygdalin- $\beta$ -cyclodextrin shows a higher SOD-like activity compare with pure AMG. As a



**Fig. 8** Effect of pure AMG and of amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation, on the growth of the  $\Delta$ sod1 mutant against free radicals produced by H<sub>2</sub>O<sub>2</sub> (disk at the top of each Petri disk) and menadione (disk at the bottom of each Petri disk).

**Table 2** The diameter of inhibition area for pure AMG and for amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation, in different concentrations, using menadione and  $H_2O_2$ .

	Pure AMG Diameter of the inhibition area (cm)		Freeze-drying (fd) Diameter of the inhibition area (cm)		Kneading (kn) Diameter of the inhibition area (cm)		Coprecipitation (co) Diameter of the inhibition area (cm)	
	Menadione	$H_2O_2$	Menadione	$H_2O_2$	Menadione	$H_2O_2$	Menadione	$H_2O_2$
Control (Menadione 5 mM or $H_2O_2$ 17.5%)	8	7.5	8	7.5	8	7.5	8	7.5
30	6.2	6.4	4.9	5.4	5.1	5.8	6	6.2
50	6	6.3	4.6	5.3	4.9	5.7	5.8	6.2
70	5.9	6.3	4.6	5.1	4.8	5.6	5.8	6.1

conclusion, amygdalin- $\beta$ -cyclodextrin compound obtained by freeze-drying register a higher SOD-like activity compared with the compounds obtained by kneading and coprecipitation. The lower SOD-like activity was obtained for compound obtained by coprecipitation. At the maximum concentration analyzed (70  $\mu$ M), a fraction of cells appears to have a higher protection.

The current study suggests that amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation are able to protect efficiently against superoxide anions and they could be considered as promising effective agents against toxicity of superoxide anion, improving significantly the growth of *Asod1* strain. They supply the  $Cu_2Zn_2SOD$  deficiency of the mutant. For this reason, they are potential therapeutic agents in the prevention and treatment of diseases mediated by free radicals. The most active is amygdalin- $\beta$ -cyclodextrin compound obtained by freeze-drying who owns the best potential.

### 3.4. Cytotoxicities of the complexes

Amygdalin- $\beta$ -cyclodextrin compounds obtained by freeze-drying, kneading and coprecipitation were examined for their antiproliferative properties compared to pure amygdalin. The *in vitro* cytotoxicity of amygdalin- $\beta$ -cyclodextrin compounds were tested on two human cells, a cervical line (HeLa); and a normal fibroblastic epithelial cell line (HFL1), using Cisplatin as positive control. The response was quantified using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The cells were exposed to various concentrations of each compound (ranging between 0.2 and 100  $\mu$ M) at 24 and 48 h.

The results revealed a promising profile, the amygdalin- $\beta$ -cyclodextrin compounds expressing different levels of cytotoxicity. On HeLa cell line, freeze-drying by two folds more potent as compared to coprecipitation, while kneading has an intermediate activity. The difference was already visible after 24 h, their differences became even more visible after 48 h, but they maintained the same trend. Freeze-drying has the highest cytotoxicity because it is the most soluble amygdalin- $\beta$ -cyclodextrin compounds, as the solubility test indicated. All inclusion compounds were by far more active than pure amygdalin, but the efficiency remained lower than those of Cisplatin (Table 3).

On normal fibroblast (HFL-1 line), all amygdalin- $\beta$ -cyclodextrin compounds showed a slightly lower toxicity than the toxicity found on tumor cells, but remarkably they were significantly less toxic than Cisplatin.

The antitumor activity of AMG is widely known, but its mechanism is not fully established. However, several studies already proved that HeLa cells are sensitive to AMG in a dose dependent manner, cell death being triggered by apoptotic mechanism via intrinsic pathway, as suggested by the increasing activity of Caspase-3 activity (Chen, Ma et al., 2013), downregulation of Bcl-2 and upregulation of Bax (Chang et al., 2006). In addition, AMG treated cells exhibit overexpression of JNK/c-Jun pathway, thus the JNK/c-Jun pathway is a possible mechanism of apoptosis in HeLa cells (Chen et al., 2013).

In cervical cancer cells, the effect on cell cycle proliferation was not investigated, but in several prostate cancer cell lines (Makarević et al., 2016) AMG inhibit the proliferation by blocking the tumor cells G0/G1 phase, by down-regulating the expression of cell cycle proteins CKD1, CKD2, cyclin A, and cyclin B. In colon cancer tumor cells, arresting of the cell cycle is done by inhibiting the ATP-binding cassette, exonuclease 1 (EXO1), sub-family F and topoisomerase (DNA) I (TOP1) (Park et al., 2005).

The selective toxicity exhibited by AMG on tumor cells, was previously described and it can be explained by the interaction between amygdaline and  $\beta$ -glucosidase (Song and Xu 2014). This interaction inhibits the cytochrome *c* oxidase, which prevent the synthesis of adenosine triphosphate and enhance the production of hydrocyanic acid. The hydrocyanic acid exerts selective toxicity on tumor cells, which, unlike nor-

**Table 3** IC<sub>50</sub> values for pure AMG and for amygdalin- $\beta$ -cyclodextrin obtained by freeze-drying, kneading and coprecipitation and for Cisplatin on human cervical carcinoma line (HeLa and a normal fibroblastic epithelial cell line (HFL1) (versus untreated cells) (mean  $\pm$  SD) (n = 3).

Cells	Compound	IC50 ( $\mu$ M)	
		24 h	48 h
HeLa cells	<b>Pure AMG</b>	231.19 $\pm$ 22.87	51.30 $\pm$ 12.78
	coprecipitation	113.93 $\pm$ 12.65	22.91 $\pm$ 8.87
	kneading	82.60 $\pm$ 15.78	17.24 $\pm$ 4.87
	Freeze-drying	63.81 $\pm$ 9.43	8.32 $\pm$ 1.09
	<b>Cisplatin</b>	22.13 $\pm$ 0.04	5.83 $\pm$ 0.15
HFL1 cells	<b>Pure AMG</b>	278.19 $\pm$ 24.18	73.08 $\pm$ 8.08
	Coprecipitation	151.15 $\pm$ 11.04	28.82 $\pm$ 6.45
	Kneading	143.21 $\pm$ 8.12	24.66 $\pm$ 4.05
	Freeze-drying	116.21 $\pm$ 5.34	24.28 $\pm$ 1.07
	<b>Cisplatin</b>	12.96 $\pm$ 1.05	2.95 $\pm$ 0.17

mal cells, lack rhodanese, an enzyme able to remove it (Li et al., 2018).

In this study we demonstrated that amygdalin- $\beta$ -cyclodextrin obtained by freeze-drying has a superior antitumor activity on HeLa cell line comparative with the other inclusion compounds and pure amygdalin.

#### 4. Conclusions

Presence of benzaldehyde molecule into the amygdalin structure induce an analgesic action, and concomitant the presence of hydrocyanic acid induces an anti-neoplastic action. Amygdalin have a low solubility in water and one possibility to increase his bioavailability is represented by his inclusion in cyclodextrins.

Three complexes were synthesized by the inclusion of amygdalin with  $\beta$ -cyclodextrin through three methods (kneading, coprecipitation, freeze-drying) using molar ratio 1:1 and after were characterized with FTIR spectrometry, X Ray-diffraction and differential scanning calorimetry. Solubility test indicate an increase of amygdalin solubility after inclusion into the  $\beta$ -cyclodextrin, being in concordance with the results obtained for in-vivo test.

Amygdalin has a clear pharmacological activity, but there is still little in depth research on the pharmacological mechanism of the compound, so it has an important application value to systematically investigate the mechanism of amygdalin pharmacological activity and develop antitumor drugs. Therefore, AMG- $\beta$ -CD compound provided by **fd** requires future testing, as a potential selective anticancer agent.

AMG- $\beta$ -CD compound obtained by freeze-drying register a higher SOD-like activity compared with AMG- $\beta$ -CD compounds obtained by kneading and coprecipitation. The lower SOD-like activity was obtained for AMG- $\beta$ -CD obtained by coprecipitation. At the maximum concentration analyzed (70  $\mu$ M), a fraction of cells appears to have a higher protection. AMG- $\beta$ -CD compound obtained by freeze-drying has the best SOD-like activity and cytotoxicity on HeLa tumor cells, so it may be considered as potential therapeutic agent.

#### 5. Contributions

Eleonora Marian, Narcis Duteanu, Laura Vicas designed the research, Tunde Jurca, Gerlinde Rusu, Mariana Muresan, Otilia Micle, Adriana Corina Hangan, Roxana Liana Stan, Corina Ionescu, Bogdan Sevastre, Eموke Pall performed experiments, Eleonora Marian and Narcis Duteanu wrote the manuscript.

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