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PhD. THESIS

**BIOSYNTHESIS AND *THYMUS VULGARIS*
EXTRACT-BASED FORMULATIONS IN THE
APPROACH TO BREAST CANCER: *IN VITRO*
EXPERIMENTAL STUDIES**

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PhD THESIS

BIOSYNTHESIS AND *THYMUS VULGARIS* EXTRACT-BASED FORMULATIONS IN THE APPROACH TO BREAST CANCER: IN VITRO EXPERIMENTAL STUDIES

ABSTRACT

Breast cancer is one of the most common types of cancer in the world and for this reason it has begun to be studied more and more intensely. Due to the morphological diversity specific to each type of cancer, current therapies have become less effective and intratumoral heterogeneity has been correlated with an increase in resistance to standard treatments. These treatments have multiple side effects which also leads to the need to develop new treatments to minimize them and increase their effectiveness. In this sense, the testing of isolated compounds from medicinal plants has become of great interest in order to modernize and optimize antitumor treatment.

Today, natural and traditional herbal treatments have become increasingly important, proving their value in many medical fields. The evaluation of plant extracts from the point of view of antitumor activity is a topical issue in the literature, especially due to the confrontation with an increase in the resistance of therapeutic agents. The natural compounds extracted from medicinal plants represent a promising starting point in numerous scientific studies in the medical field, especially in the antitumor sphere, presenting antiproliferative and proapoptotic activity, aspects that are highlighted in in vitro and in vivo studies on different types of cancers. Numerous antitumor agents have been described in various types of breast cancer. Among the best known herbs are Thymus species, used since the past as a traditional treatment and correlated with antimicrobial, antiseptic and more recently antitumor activity.

PURPOSE OF THE RESEARCH

In the present thesis, the identification of new natural compounds with antineoplastic activity in order to broaden the horizon of antitumor medication and to improve current treatment was widely evaluated. In this regard, was studied the species *Thymus vulgaris*, which belongs to the genus *Thymus* and is part of the large family *Lamiaceae*. It has an important therapeutic activity and is a rich source of medicinal compounds (polyphenols, flavonoids, flavonols and tannins). The cytotoxic activity of the total aqueous and hydro-alcoholic extract of *Thymus vulgaris* was tested on healthy cell lines (mesenchymal stem cells, immortalized human keratinocytes and murine epidermis) and tumors (skin and breast). Due to the stability and low solubility in aqueous solutions, the use of pure extracts in the pharmaceutical field is limited.

The main purpose of this paper was to evaluate the role of β - and γ -cyclodextrins and silver nanoparticles in order to improve the biopharmaceutical properties of the tested extracts.

THE GENERAL PART

The general part is structured in three important chapters. The first chapter presents general aspects about the type of cancer studied - breast cancer and highlights the current status of knowledge in the literature. In addition to the general aspects that characterize this tumor form, are also presented the risk factors, incidence and classification, progression, etiopathogenic mechanisms involved in the development of breast cancer and aspects related to current treatment and perspective. The second chapter contains general data on biologically active compounds isolated from medicinal plants and their use in the pharmaceutical field, then continuing with the presentation of their antioxidant and antitumor activity on many tumor cell lines. Finally, the emphasis was on antitumor activity in breast cancer (two lines of breast cancer were studied) highlighting the cell-induced effects in vitro / in vivo of these compounds. The last chapter describes general aspects in the field of nanotechnology, more precisely aspects related to silver nanoparticles, their chemical, physical and especially biological synthesis as well as their pharmacological activity in the medical field.

THE SPECIAL PART

The special part includes the experimental part in which the materials and methods used are described as well as the results and discussions part. It is also divided into three major chapters. The conclusions are presented at the end.

The first chapter addressed issues related to biosynthesis and characterization of different formulations based on *Thymus vulgaris*: total aqueous and hydro-alcoholic extract of *Thymus vulgaris* (TV_AE and TV_HAE), encapsulation of extracts in β and γ cyclodextrins with the formation of inclusion compounds (TV_ β CD and TV_ γ CD) and obtaining silver nanoparticles by biosynthesis with *Thymus vulgaris* extracts (AgNPs_TV_AE and AgNPs_TV_HAE).

The two total extracts of *Thymus vulgaris* were prepared according to well-defined protocols in the literature. They were then subjected to physico-chemical characterization by spectrophotometric methods in order to establish the composition and evaluate the biologically active properties. The polyphenolic compounds of the extracts were detected by HPLC-MS chromatographic technique and it was shown that rosmarinic acid, rutoside, kaemferol but also caffeic acid and quercetin were identified as the main compounds in the total hydroalcoholic extract of *Thymus vulgaris* as opposed to the aqueous extract. where rosmarinic acid is the main compound, followed by eriocitrin, luteolin, apigenin, quercetin and some forms of glycosylated flavonoids.

The inclusion compounds were synthesized by encapsulating the extracts in β and γ cyclodextrins in a molar ratio of 1: 2, and the formation of supramolecular systems was confirmed by SEM, DSC and FTIR techniques. Because the aqueous extract had a poorer composition in biologically active principles, only the total hydroalcoholic extract of *Thymus vulgaris* was encapsulated in cyclodextrins. Regarding the synthesis of silver nanoparticles with the two extracts, the procedure using plant products - in this case the aqueous and hydro-alcoholic extract of *Thymus vulgaris* - was used, the so-called "green synthesis". The formation and stability of nanoparticles was confirmed by three working techniques TEM, DLS and potential Zeta.

The second chapter presents the screening evaluation of the cytotoxic activity of the compounds synthesized and characterized in the first chapter on different tumor cell lines: squamous cell carcinoma (A431), human melanoma (A375), murine melanoma (B16 4A5), human

breast carcinoma (MCF 7 and MDA-MB-231) but also on three healthy cell lines: mesenchymal stem cells (MSC), immortalized human keratinocytes (HaCaT) and murine epidermis (JB6Cl41-5a) using different concentrations.

Evaluation of the in vitro antiproliferative effect of aqueous and hydro-alcoholic extract of *Thymus vulgaris* (at different concentrations) and of the mentioned formulations: silver nanoparticles obtained by biosynthesis using aqueous extract, respectively alcoholic thyme (also at different concentrations) and complexes with Cyclodextrins of thyme hydroalcoholic extract was performed after a 24-hour stimulation by the Alamar blue cell viability test.

Regarding healthy cell lines, it was found that immortalized human keratinocytes were not significantly influenced, on the mesenchymal cells the silver nanoparticles and cyclodextrin complexes of the two extracts exerted cytotoxic effects at the highest concentrations tested, and murine epidermal silver nanoparticles also at the highest concentrations exerted cytotoxic effects. It should be noted that the silver nanoparticles obtained with aqueous extract proved to be more stable over time and with a constantly beneficial action.

In the case of tumor lines, a representative cytotoxic effect for TV_AE was observed on the MCF 7 cell line at the highest concentration tested, in contrast to the MDA-MB-231 cell line where viability was not significantly altered. In contrast, exposure of cell lines to TV_HAE is correlated with a considerable decrease in viable cells on both tumor lines in a dose-dependent manner, especially in the case of MDA-MB-231 cells. Viability on the A431 and A375 cell lines was not visibly affected, there is even a slight increase in the A375 line following the interaction with the two extracts. An important aspect is that following the exposure of MCF 7 cells to the action of silver nanoparticles obtained with aqueous extract the number of viable cells decreased drastically for all tested concentrations, in a dose-dependent manner, but in the case of cell line MDA-MB-231 no showed cytotoxic activity. A decrease in the percentage of viability was also noticed for lines A431 and A375, but not in a significant way. Stimulation of human breast cancer cells with TV, TV_βCD and TV_γCD was performed under the same conditions as that applied to normal cells, to evaluate the cytotoxic activity of the compounds (at min. Three different concentrations) for 24 hours. On the MDA-MB-231 line the most pronounced effect was attributed to the TV_γCD complex. In the case

of the MCF 7 cell line, the most intense antiproliferative effect was obtained for TV_βCD.

The last chapter aimed at a more detailed evaluation of the behavior of MCF 7 and MDA-MB-231 cells in the presence of said compounds. Following the use of the MTT test, the viability percentages obtained by stimulating the cells with TV, TV_βCD and TV_γCD did not show significant differences compared to the viability percentages obtained when using the Alamar blue test. TV_βCD and TV_γCD denoted significant cytotoxic activity on MCF 7 and MDA-MB-231 cells, with the most notable activity of the TV_γCD complex on MDA-MB-231 cells. Also, silver nanoparticles contribute to the improvement of the antiproliferative effect in a dose-dependent manner on the MCF 7 cell line, in the case of the MDA-MB-231 line the effect being a stimulating one.

By exposing healthy HaCaT cells in the presence of TV and TV_βCD no effect was observed on the ability of cells to migrate and proliferate, on the contrary their stimulation with TV_γCD intensified cell migration in vitro. Regarding breast tumor cells, it is observed that the migratory activity of MCF 7 cells was slightly inhibited by the action of TV_βCD, the influence of TV compounds and TV_γCD on them being insignificant. It should be noted that the migratory effect on the MDA-MB-231 cell line was less intense after exposure to TV and TV_βCD compared to the control, the lowest migration rate being noted for the compound TV_γCD. These results are consistent with those obtained when assessing cell viability.

In order to evaluate the in vitro apoptotic degree of MCF 7 and MDA-MB-231 cells after treatment with TV, TV_βCD and TV_γCD resulted in a significant apoptotic percentage for the highest concentration tested (100 μg / mL), as follows: pronounced apoptotic effect occurred after exposure of MDA-MB-231 cells to TV_γCD, whereas exposure of MCF 7 cells to TV_βCD action is correlated with a weaker apoptotic effect.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

The results of the experiments in this doctoral thesis highlight the antitumor activity of cyclodextrin complexes of *Thymus vulgaris* extracts and biosynthesized silver nanoparticles. Following the screening, an increased activity was observed against breast cancer cells, these being the most affected by the tested compounds. These

formulations therefore have promising effects on breast cancer cells. In the case of the tested tumor lines, the most pronounced cytotoxic activity was observed in breast cancer cells (MCF 7 and MDA-MB-231). The extracts decreased cell viability at the highest concentrations on both lines of breast adenocarcinoma.

After stimulation of MCF 7 cells with aqueous colloidal solution of silver nanoparticles, there was a drastic increase in the antiproliferative effect compared to the pure extract for all concentrations tested with very low viability values.

Also, by incorporating the total hydro-alcoholic extract of *Thymus vulgaris* in β - and γ -CD, the cytotoxic effect was optimized compared to the pure extract on both breast cancer cell lines (MDA-MB-231 and MCF 7). Therefore, the sensitivity and stability of the compounds were improved, resulting in the optimization of the *in vitro* antiproliferative property. Molecules extracted from plant plants are therefore potential antineoplastic candidates in the treatment of breast cancer. The combination of natural compounds with standard chemotherapeutics is a promising approach in the field of antitumor and detailed studies are needed to explore the potential.