Thyme Leaves Aqueous Extract and its Formulations

A comparative study based on chemical structures and biological activity

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The present study was conducted in order to evaluate the biological activity of thyme aqueous extract as compared to the silver nanoparticles obtained on it on four different cancer lines: non-melanoma skin cancer (A431 cell line), melanoma skin cancer (A375cell line), hormone dependent breast cancer (MCF-7 cell line), and non-hormone dependent breast cancer (MDA-MB-231 cell line). Of particular importance in the study of biological activity is the chemical composition. Formulas rich in compounds of classes like those of flavonoids are recognized for their efficacy and important antioxidant effect. The extract tested at various concentrations ranging from 10-1000 µg/mL exhibited a pronounced effect only on MCF-7 cells at the highest concentration tested and regarding the biosynthesized silver nanoparticles, the effect can be noted even at 1 ìM also only in the case of MCF-7 cells.

Keywords: thyme aqueous extract, green synthesis, silver, cytotoxicity

Medicinal plants are recognized for their beneficial effects and have been used since antiquity by various peoples. About 3500 years ago *Thymus vulgaris* was first known for its aromatic effects and then its analgesic and anti-inflammatory effects in various cutaneous diseases were proven [1,2]. It is also noticeable by its expectorant effect and its utility in gastrointestinal disorders. Because this plant belongs to the Lamiaceae family, both oil and thyme extract have a large number of hydroxyl groups, which is why they have a strong antioxidant effect. Two phenolic compounds namely thymol and carvacol are found in high concentrations in the essential oil of *Thymus vulgaris* (thyme) as also in the different species of Thymus [3]). In some studies it has been demonstrated that *Thymus vulgaris* has antiproliferative activity on a number of tumor cells, such as squamous and cervical tumor cells [2]. Nanotechnology has become increasingly necessary and used in oncology therapy because classical chemotherapy has proved to be quite disadvantageous; has high toxicity, the concentration of therapeutic agent reaching the tumor site is minimal and resistance often occurs, which is why limiting the use of chemotherapies is attempted. Nanoparticles are used to streamline the administration of anticancer agents to malignant cells [4]. Imaging techniques using fluorescent nanoparticles targeted to a variety of human cancers are studied to allow for the location of diseased cells *in vivo*. Also, are important the pathologies associated which increase the risk for patients [5]. There are hopes that modern techniques will improve the accuracy of diagnosis in many types of imaging modalities used to detect and monitor the maladies [6]. These techniques may also allow early detection of cancer among the high-risk population and guide the duration and type of therapy in patients with more advanced stages of different diseases[7]. At present, high interest in antitumoral research is the synthesis of silver nanoparticles (SilNPs) and their association with various extracts in order to obtain formulations with increased efficacy. The application of this type of nanoparticles in the medical field is not limited to increasing the anti-tumor effect, but also for improving the anti-oxidant, anti-microbial, anti-thrombotic effect or can be used as catalysts [8,9]. The method of obtaining the most efficient and non-toxic nanoparticles with a minimum energy consumption is the biological one through a process called green synthesis.

The aim of the present research was to compare the biological activity exerted by thyme aqueous total extract (TV) and silver nanoparticles obtained by biosynthesis. The experiments were conducted on different human tumoral cell lines: skin cancer (A431), melanoma (A375), and breast cancer (MCF-7 and MDA-MB-231).

Experimental part

Materials and methods

All the substances utilized in the current study were of analytical grade and purchased from Sigma Aldrich, Germany. Silver nitrate was purchased from Merck, Germany.

Thyme (*Thymus vulgaris*) was harvested from the west of Romania and provided from a local marked. The plant material has been verified and certified by faculty specialists and a voucher specimen is kept at the Herbarium of the Faculty of Pharmacy, Victor Babes University of Medicine and Pharmacy Timisoara, Romania.

Thyme dried leaves (2.5 g) were boiled in de-ionized water (50 mL), cooled and then filtered. A part of the fresh extract was immediately used for the preparation of silver nanoparticles and the other part was lyophilized and kept in optimal conditions for further characterization and testing studies.

To a solution of 1 mM silver nitrate (10 mL) was added thyme aqueous extract (10 mL) and with de-ionized water the final volume was adjusted to 50 mL. The solution was shaken in an orbital shaker (250 rpm, room temperature, 72h) until the colour was changed from light-brown to yellow.

Identification of phenolic structures was realized on a Shimadzu device (SPD-10A UV and LC-MS 2010 detectors, EC 150/2 NUCLEODUR C18 Gravity SB 150 x 2mm x 5µm column on the following conditions: two mobile phases - water and acetonitrile, both acidified with formic acid, at room temperature, flow rate 0.2 mL/min, and gradient

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programme 0.01-20 min. 5% (ACN/HCOOH), 20.01-50 min 5-40% (ACN/HCOOH), 5-55 min, 40-95% (ACN/HCOOH), 55-60 min 95% (ACN/HCOOH). All reagents and solvents used were analytical grade chemicals. Physico-chemical characterization of silver nanoparticles was realized by well-known methods on specific devices: spectroscopy, dynamic light scattering, transmission electron microscopy and zeta potential.

The tumoral cells utilized in the current research were: A431 - nepidermoid carcinoma (ATCC® CRL-1555™), A375 -melanoma (ATCC® CRL-1619™), MCF-7-adeno-carcinoma (ATCC® HTB-22™), and MDA-MB-231 - adenocarcinoma (ATCC® HTB-26™). Cell procurement was made from the company American Type Culture Collection, packaged in frozen vials and stored in liquid nitrogen until the beginning of the experiments.

For cultivation of A431, A375 and MDA-MB-231 cells the culture medium was removed, the cell layer was gently rinsed with 0.25% trypsin and EDTA solution to eliminate all traces of serum containing trypsin inhibitor. Trypsin-EDTA solution was added to the vial and the cells were monitored on an inverted microscope until they were dispersed. Full growth medium was then added and the cells were aspirated by mild pipetting, then incubated at 37°C. The basic medium for these is the Dulbecco's Modified Eagle's Medium, to which fetal bovine serum has been added to a final concentration of 10%. Cultivation of MCF-7 cells involves performing the same steps with the reference that the basic medium for this cell line is the Essential Minimal Eagle medium to which the following components are added: recombinant human insulin 0.01 mg / mL; fetal bovine serum to a final concentration of 10%.

The Alamar blue cytotoxicity assay was performed in 96-well plates. The volume of reagent added to each well was 10 μL. The plates were then incubated at 37°C for 1-4 h. The measurements were made on the spectro-photometer and the absorbance was measured at 570 nm and 600 nm [10]. To minimize errors, 4 to 8 replicates were used for each sample from which the arithmetic mean was later deduced.

Results and discussions

The results of the chromatographic analysis indicated the presence of different biologically active compounds. table 1 lists the major compounds identified in the thyme aqueous extract. Rosmarinic acid is an identified dominant component. Other components identified were eriocitrin, luteolin, apigenin, quercetin flavonoids and some of the glycosylated forms.

Tabel 1
CHEMICAL COMPOUNDS IDENTIFIED IN THE THYME AQUEOUS EXTRACT

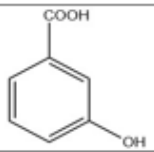
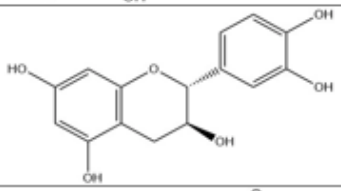
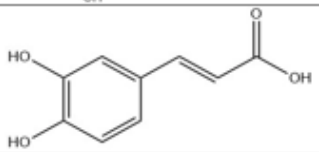
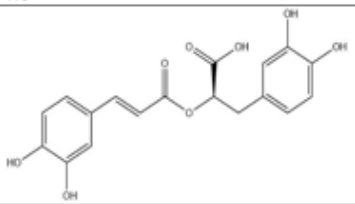
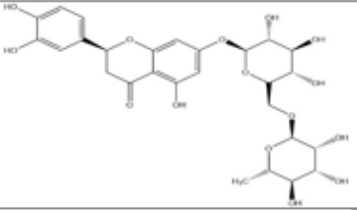
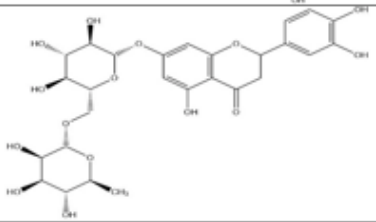
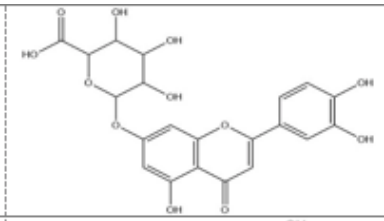
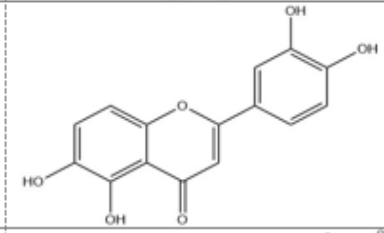
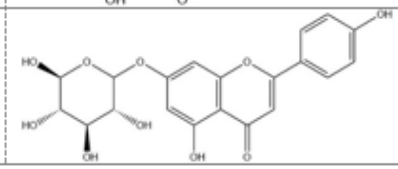
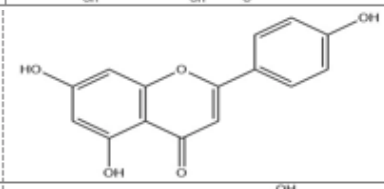
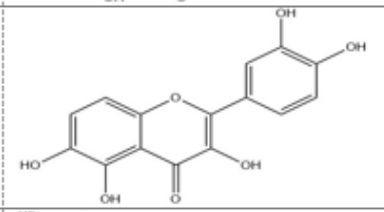
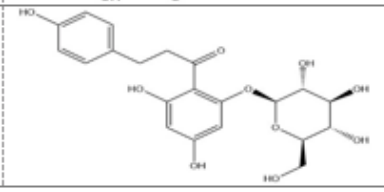
Compound name	Compound structure	Molecular formula	Molecular weight
p-Hydroxybenzoic acid		$C_7H_6O_3$	138.12 g/mol
Catechin		$C_{15}H_{14}O_6$	290.26 g/mol
Caffeic acid		$C_9H_8O_4$	180.16 g/mol
Rosmarinic acid		$C_{18}H_{16}O_8$	360.31 g/mol
Eriocitrin		$C_{27}H_{32}O_{15}$	596.538 g/mol
Luteolin-7-O-rutinoside		$C_{27}H_{30}O_{15}$	594.522 g/mol

Table 1 - Continuated

Luteolin-7-O-glucuronide		$C_{21}H_{18}O_{12}$	462.363 g/mol
Luteolin		$C_{15}H_{10}O_6$	286.24 g/mol
Apigenin-7-O-glucoside		$C_{21}H_{20}O_{10}$	432.381 g/mol
Apigenin		$C_{15}H_{10}O_5$	270.24 g/mol
Quercetin		$C_{15}H_{10}O_7$	302.236 g/mol
Phloridzin		$C_{21}H_{24}O_{10}$	436.413 g/mol

Sample	*Technique*	*Data*
SilNPs	TEM	92 nm ± 5 (S.D.), majority spherical shape
SilNPs	DLS	132 nm ± 4 (S.D.) in deionized water, 145 nm ± 5 (S.D.) in culture media
SilNPs	Zeta	-25 mV in deionized water, - 20 mV in culture media

Table 2
CHARACTERIZATION OF SILVER NANOPARTICLES OBTAINED BY BIOSYNTHESIS

The SilNPs were characterized by well-known methods, and the data regarding their characteristics are presented in table 2.

In order to quantify the antiproliferative activity of modern compounds with antitumoral potential, researchers use the Alamar Blue reagent to detect the metabolic activity of the cells. Resazurin is the active substance of the reagent. It is a blue redox indicator, devoid of toxicity that has the ability to easily penetrate inside the cell. Viable cells have metabolic activity and in this case resazurin produces a color change: it is transformed by the reduction process in resorufin, a red compound that prints the cells a fluorescent character. Instead, if the cell is dead, the reduction process does not take place, and the cells retain their blue color and do not emit fluorescence. The higher the cell viability, the higher the color intensity and the fluorescence of the peritumoral environment. The existence or absence of cellular metabolic activity is therefore an indicator of cell viability or viability.

The compounds, were tested in terms of their efficacy on cell viability on two skin cancer cells, A431 and A375. The results of the thyme extract and silver nanoparticles on cells, after 24h stimulation is presented in figure 1. Data indicate that the extract, tested in concentrations between 10-1000 µg/mL, exhibited a slight effect on the cell viability of the two skin cancer cell lines: a slight decrease in viability of A431 cells at the lowest concentrations, respectively a stimulatory effect on A375 cells at the highest concentrations. Regarding silver nanoparticles, at the highest concentration tested in both cell lines can be observed the reduction of viability.

In a study by Arora et al., it was clearly demonstrated that the morphology of A431 and HT-1080 cells was not influenced by the silver nanoparticles used up to a concentration of 6.25 g / mL, and an abnormal size, contraction and the rounded appearance of the cells could be noticed at high concentrations [11]. In another study performed on A431 cells, the following data were obtained with respect to the IC50 value of 82.39 ± 3.1, 83.57 ± 3.9 and 78.58 ± 2.7 and 68.71 ± 3.1 µg / mL for three different types of biosynthetic silver nanoparticles and commercially

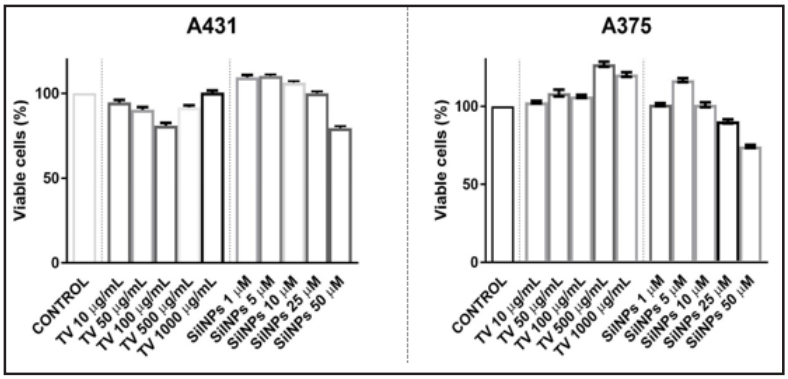


Fig. 1. The percentage of viable tumoral cells after stimulation with different concentrations of thyme aqueous extract and biosynthesized silver nanoparticles

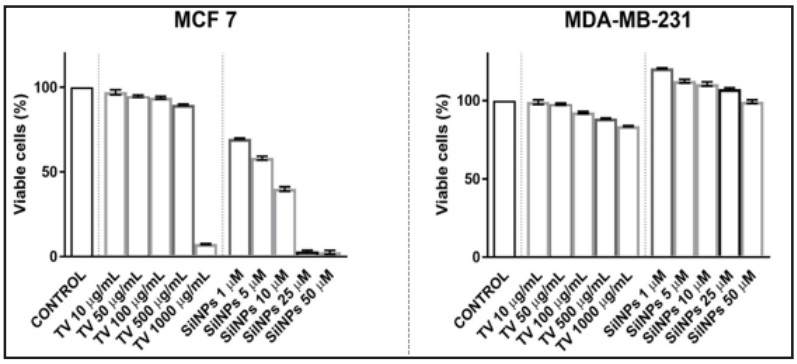


Fig. 2. The percentage of viable breast cancer cells after stimulation with different concentrations of thyme aqueous extract and biosynthesized silver nanoparticles

available silver nanoparticles. The results clearly highlight the dose-dependent toxicity of silver nanoparticles [12].

It was also checked the impact of extract and its silver nanoparticles on breast cancer cells viability by the means of Alamar blue assay. As it can be observed in figure 2, TV induced a marked decrease of MCF7 cells viability only at the highest concentration tested -1000 g/mL, whereas in the case of SilNPs the effect can be noted even at 1µM.

Regarding the activity of thyme extract and its biosynthesized particles with silver, T47D cells were stimulated with different concentrations of test compounds samples (concentrations between 12.5 -200µg/mL) in one study conducted by Heidari et al. Their results showed that SilNPs affected the cell viability in a percentage of about 90% at the highest concentration of 200 µg/mL, while the thyme extract was less toxic on same cells [13,14].

Conclusions

In this study, an aqueous extract of thyme was obtained which was later used to produce silver nanoparticles by biosynthesis. Cytotoxic activity was tested on four tumor lines: 2 skin cancers and 2 breast cancers. The results indicated a pronounced effect of silver nanoparticles, in a dose-dependent manner, on the viability of hormone dependent breast cancer cells.

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