Genistein and its Fatty Acid Esters as New *In vitro* Antitumor Compounds

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This present study describes the biological activity of genistein and genistein derivatives with ester structure on two tumor cell lines, A431 (skin epidermoid carcinoma) and A375 (human melanoma). Genistein esters were synthesized following the pathway of chemical esterification which involved solvent medium, triethylamine and fatty acid chloride. Esterification of genistein leads to increased hydrophobicity in order to have a better absorption while the compounds cross the membrane barrier.

Keywords: genistein, esters, antiproliferative activity, A431, A375

The therapeutic benefits of plant derived products are well known since antiquity, therefore current research studies are focused on natural compounds in order to obtain new derivatives with better biological activity and significantly reduced side effects. Natural compounds are generally modified for the purpose of increasing their low stability and poor solubility, as well the resistance against oxidation and other metabolic processes occurring in the bloodstream. Fatty acids are natural compounds often used to obtain stable derivatives of natural bioactive compounds.

Flavonoids, a large class of bioactive compounds, are secondary plant metabolites and can be found in fruit and vegetable. Genistein (4',5,7-trihydroxyisoflavone) belong to the sub-class of flavonoids, named isoflavones and is the major active compound from soybean (*Glycine max* Family *Fabaceae*). In the present, genistein is the subject of many studies, being know as inhibitor for protein tyrosine kinase, and currently tested for the angiogenesis inhibiting activity [1-3]. The anticancer mechanism of this isoflavone include the inhibition of topoisomerase II, phosphatidyl-inositol turnover, ABC transporters, and other proteins and induce apoptosis, suppress lymphocyte activation, inhibits cell proliferation, angiogenesis, and the production of reactive oxygen species [4-8]. Further, the chemical structure of genistein is similar to that of mammalian estrogens, and the modulation of the actions mediated by estrogen receptors, make this compound a phytoestrogen [9-11]. Our research team studies involved the changes occurring in the anti-inflammatory activity of genistein when she's incorporated in cyclodextrin [12], effect on tumor size, metastasis potential and melanization in B16 model of murine melanoma of genistein [13] and the potential as antiproliferative agents of genistein esters on Mesenchymal stem cells [14].

The aim of this research was to obtain and evaluate the biological activity of genistein esters comparative to the native compound.. The esters were subjected to preliminary *in vitro* tests as antitumor agents, by means of MTT assay, on the tumor cell-lines, A431 (skin epidermoid carcinoma) and A375 (human melanoma).

Experimental part

Materials

Genistein was acquired from Extrasynthese (France, purity >95%), myristoyl chloride, palmitoyl chloride and stearoyl chloride were purchased from Sigma Aldrich, triethylamine from Merck, and all the other chemicals including the solvents (chloroform, ethyl acetate, methanol, acetonitrile), were at least of analytical grade and used as received. All the solvent mixture was defined as v/v.

Chemical esterification of genistein

Genistein (1 mmol) was solubilized in chloroform (10 mL) under magnetic stirring. The temperature was adjusted at reflux temperature and triethylamine was added to the solution. The esterification begins after the fatty acid chloride (2 mmol) was slowly dropped in the solution (fig. 1). After several hours, the mixture was cooled at room temperature, washed three times with distilled water until neutral *p*H and dried over anhydrous $CaSO_4$.

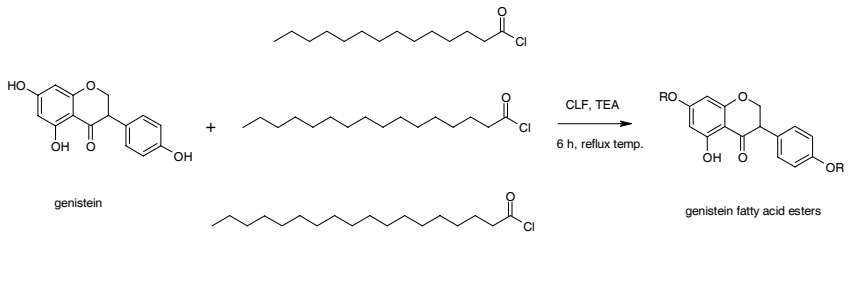

Fig. 1. Chemical esterification of genistein using: myristoyl chloride, palmitoyl chloride and stearoyl chloride, in chloroform (CLF) media, in the presence of triethylamine (TEA), for 6 h at reflux temperature; R – fatty acid chain

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The solvent was removed from the reaction mixture using a rotary evaporator in order to obtain the crude precipitate. Finally the products were purified by column chromatography to achieve a highly pure genistein ester in range between 32-38%.

The physico-chemical methods used to evaluate the formation of the desired compounds were TLC analysis and HPLC analysis and to confirm the ester structures FT-IR and MS analyses.

TLC analysis

The chemical syntheses reactions were monitored qualitatively by the help of thin layer chromatography (silica gel plates 60 F254, Merck) using different mixtures of solvents; chloroform/ethyl acetate (60/40) has been shown to be more specifically. Both genistein and their esters were visualized in ultraviolet light (254 nm).

HPLC analysis

An YL 9100 HPLC System, equipped with vacuum degasser (YL 9101), quaternary pump (YL 9110), column compartment (YL 9131) and spectrophotometric detector (YL 9120) was used for qualitative analysis of the samples. Working conditions were Nucleosil 100 C-18, 250 x 4.6 mm x mm column, particle diameter 5µm, wavelength 254 nm. Separation of components from the reaction medium, flavonoid and flavonoid derivatives, was carried out using as mobile phase water (0.1% acetic acid) / acetonitrile (0.1% acetic acid) with a flow rate 1 mL/min, at 25°C.

FT-IR spectroscopy

FTIR spectra of genistein and genistein derivatives were obtained on the Perkin Elmer SPECTRUM 100 spectrometer using the UATR technique on 4000-400 cm^{-1} spectral range.

MS spectroscopy

For NanoMate HCT MS experiments, the solution of genistein and its derivatives was prepared by dissolving the dry sample in pure methanol to a concentration of about 10 pmol μL^{-1}. Mass spectrometry was conducted on a High Capacity Ion Trap Ultra (HCT Ultra, PTM discovery) mass spectrometer from Bruker Daltonics, Bremen, Germany. All mass spectra were acquired in the mass range (200-1500) m/z, with a scan speed of 8000 m/z per second. The m/z scale of all mass spectra was externally calibrated using G2421A electrospray "tuning mix" from Agilent Technologies (Santa Rosa, CA, USA) as calibration standard. Following calibration procedure, the obtained mass accuracy was situated within the normal range of a HCT MS instrument.

Fully automated chip-based nanoelectrospray was performed on a NanoMate robot incorporating ESI 400 Chip technology (Advion BioSciences, Ithaca, USA) controlled and manipulated by ChipSoft 7.1.1 software operating under Windows system. The robot was coupled to the HCT Ultra mass spectrometer via an in-laboratory made interface. 10µL aliquots of the working sample solutions were loaded onto a NanoMate 96-well plate. The robot was programmed to aspirate 5µL of the sample, followed by 2 µL of air into the pipette tip and afterwards deliver the sample onto the inlet side of the 400 microchip. NanoMate HCT MS system was tuned to operate in the negative ion mode at -0.20 kV ESI potential and 0.85 p.s.i. nitrogen back pressure. Under these working conditions a stable ESI signal and a drastic reduction of the in-source decay were observed. The source block maintained at the constant temperature of 100°C provided an optimal desolvation of the generated droplets without the need of desolvation gas. All mass spectra were processed by Data Analysis 3.4. Software from Bruker Daltonics (Bremen, Germany), which allows signal extraction, smoothing and subtraction. Proposals for molecular ion composition were made by exact mass calculation.

MTT assay

Two tumor cell-lines were used, A431 (skin epidermoid carcinoma) and A375 (human melanoma), in order to evaluate the antiproliferative activity of the ester derivatives of genistein using as standards the native compounds, genistein. The tumor cells were preserved in minimal essential medium (Sigma Aldrich) in the presence of 10% fetal calf serum (PromoCell, Heidelberg, Germany), with the addition of 1% antibiotic mixture (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany). The cells were seeded onto a 96-well microplate and after 24 h, 200µL new medium (Gibco BRL, Invitrogen, Carlsbad, CA, USA) were added together with the tested substances (G, GMAE, GPAE and GSAE) dissolved in DMSO. The mixture was incubated 72 h in humidified atmosphere with 5% CO_2 at 37°C and after that the viability of the cells was evaluated by adding 20µL MTT (5 mg/mL).The medium was removed, after 4 h, and the precipitate formed (blue crystals), as result of the mitochondrial reductase activity, was dissolved in solubilisation solution, and then was measured the viability using spectrophotometry, at wavelength 545 nm. As control was used untreated cells and all the experiments have been achieved at least in triplicate. Solvent used for the stock solutions of the bioactive compounds (10 mM) was DMSO and a concentration of 0.1% did not have any action concerning cell proliferation.

Results and discussions

In order to find the optimal eluent for genistein derivatives separation different solvent mixtures was used: ethyl acetate/chloroform (6/4), ethyl acetate/isopropyl alcohol/water (10/1.7/1.3), toluene/chloroform/acetone (4/2.5/3.5) and chloroform/methanol/water (7/3/0.03). The best visualization was obtained when the samples have been introduced in chloroform/ethyl acetate solvent media. All the TLC analyses revealed the formation of only one derivative of genistein, as can be observed in figure 2. Genistein and the fatty acids were dissolved in 2 mL chloroform for the TLC analysis and the other samples were taken from the reaction media. The detection was carried out directly at UV-254 nm and the volume applied was 5 µL. The absorption of samples applied to the plate can be observed in the case of genistein and its derivatives but the fatty acid do not appear in UV light.

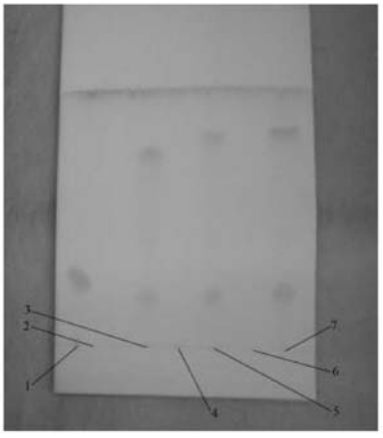

Fig. 2. TLC chromatogram: 1 – genistein; 2- myristic acid ; 3 – GMAE (genistein myristic acid ester) ; 4 - palmitic acid; 5 – GPAE (genistein palmitic acid ester); 6 – stearic acid; 7 – GSAE (genistein stearic acid ester)

Compound	Rf values	Retention time (min)
Genistein	0.26	16.8
GMAE	0.71	19.5
GPAE	0.76	21.4
GSAE	0.85	23.2

Table 1
RF VALUES AND RETENTION TIMES FOR GENISTEIN AND THEIR DERIVATIVES

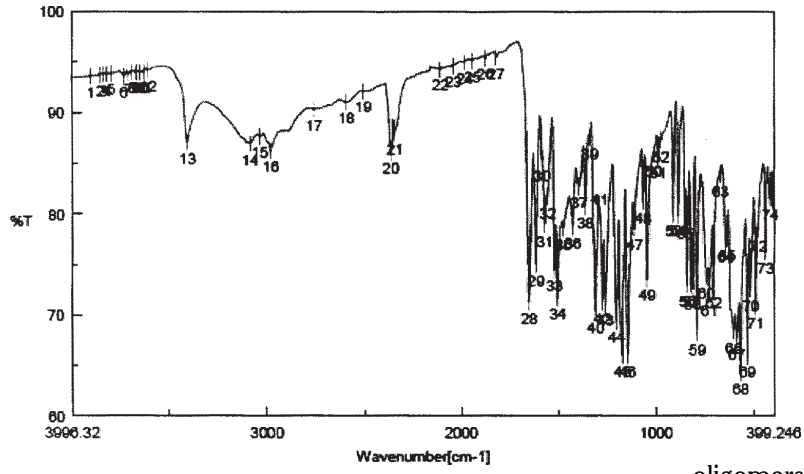

Fig. 3. FT-IR spectra of genistein using the UATR technique on 4000-400 cm^{-1} spectral range

The solid products obtain after separation and recrystallization were dissolved in methanol for the HPLC analyses. The chromatograms showed only one peak assigned to corresponding esters of genistein in each case. The results of the analyses, Rf values and retention time are presented in table 1.

The structures of genistein myristate, genistein palmitate and genistein stearate were elucidated using FT-IR and MS spectra. The formation of the esters is confirmed by the IR spectral data. The products presented the signals corresponding to the band of the carbonyl group of the ester in all cases: 1755.14 cm^{-1} for GMAE, 1732.12 cm^{-1} for GPAE and 1738.01 cm^{-1} for GSAE. The vibrations of carbonyl groups of fatty acids at 1703.06 cm^{-1} disappear in the spectra of derivatives, but the specific carbonyl band of genistein at 1649.06 cm^{-1} can be found in all derivatives spectra. In figures 3 and 4 are presented FT-IR spectra for genistein and for the compound obtain after chemical esterification with myristoyl chloride, respectively.

Inspection of the screening MS spectrum in the negative ion mode presented in figure 5 indicates the formation with high intensity of the most abundant singly charged ions related to genistein as follows: along with the monodeprotonated ion $[M-H^+]^-$ at *m/z* 269.30, two oligomers, due to the high sensitivity of the method, were found. Thus, the dimer detected as the singly charged ion $[2M-H^+]^-$ at *m/z* 539.45 and the trimer detected as the singly charged ion $[3M-H^+]^-$ at *m/z* 809.77 were also formed. The oligomerization of this small molecule is a typical phenomenon occurring in solution detected by the high sensitivity of NanoMate HCT MS, even though the concentration was at a rather low value of 10pmol μL^{-1}. For genistein derivatives were found the values $[M-H^+]$ at 688.98 *m/z* (GMAE); $[M-H^+]$ at 748.8 *m/z* (GPAE) and $[M-H^+]$ at 804.96 *m/z* (GSAE).

The ion species described above were accompanied by alkali adducts, monochlorinated adducts respectively. This phenomenon occurred both for the molecular species $[M+Cl^-]^-$ detected at *m/z* 305.72 and also for the dimer species $[2M+Cl^-]^-$ detected at *m/z* 575.65. The process of chloride adduct formation is typical for ESI MS in the negative ion mode.

From the assessment of the MS spectrum presented in figure 5 we can conclude that the optimized ionization and detection conditions were ideal to generate straightforward molecule identification.

A useful tool for the preliminary evaluation of the antiproliferative activity of bioactive compounds is MTT assay. The solvent routinely used for *in vitro* experiments is

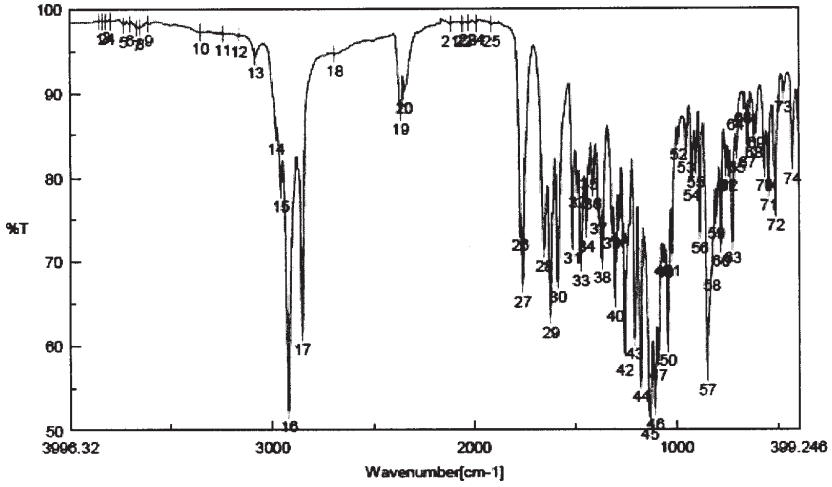

Fig. 4. FT-IR spectra of GMAE using the UATR technique on 4000-400 cm^{-1} spectral range

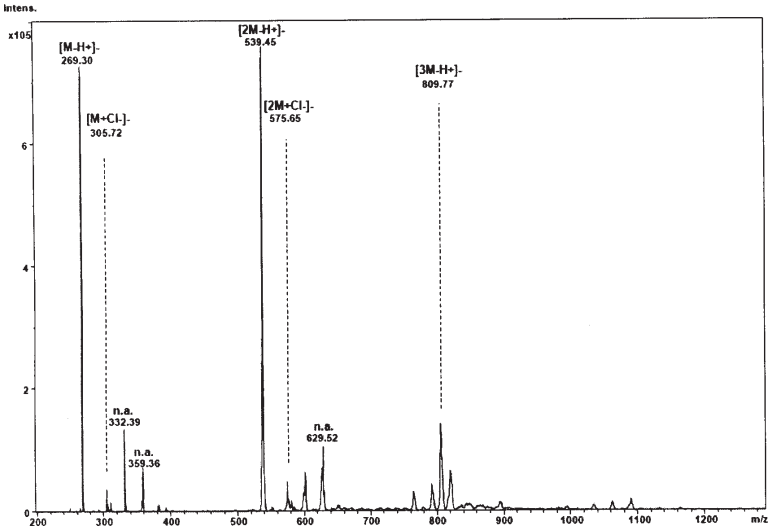

Fig. 5. Fully automated (-) nanoESI chip HCT MS of the genistein sample solution. Solvent: MeOH; sample concentration 10 pmol/μL; acquisition time 1 min; nanoESI chip: -0.20 kV; capillary exit: -50 V

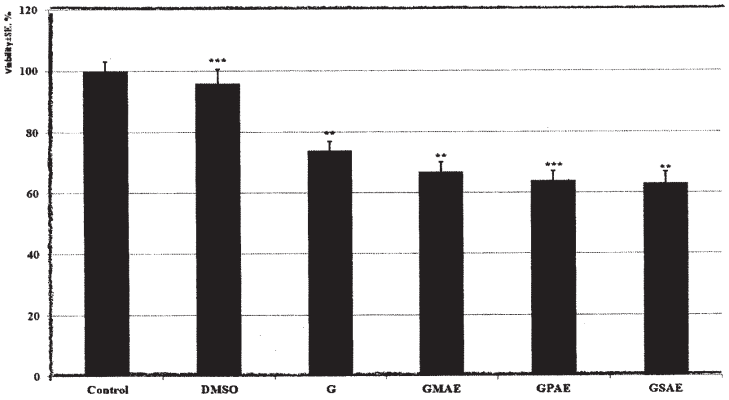

Fig. 6. MTT assay on A431 tumor cell lines for genistein (G), genistein myristic acid ester (GMAE), genistein palmitic acid ester (GPAE) and genistein stearic acid ester (GSAE)

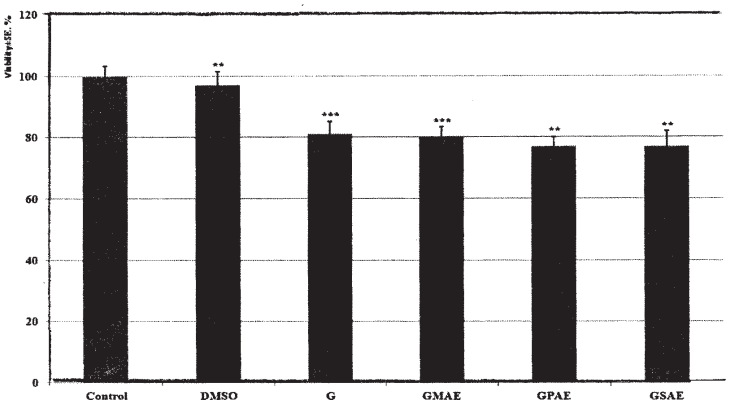

Fig. 7. MTT assay on A375 tumor cell lines for genistein (G), genistein myristic acid ester (GMAE), genistein palmitic acid ester (GPAE) and genistein stearic acid ester (GSAE)

DMSO because has an insignificant influence on cell viability when is used in small concentration (≤ 0.1%). As positive control for the viability assay were considered the untreated cells. Our data showed that all genistein derivatives tested exhibited a strong antiproliferative activity on both tumor cell lines, but in the case of A431 (skin epidermoid carcinoma) tumor cell lines the activity was more pronounced as compared to A375 (human melanoma cells), difference shown in figures 6 and 7; the results were expressed as the mean ± standard deviation. One way Anova followed by Bonferonni–Dunn post-hoc test was used to determine the statistical difference between various experimental and control groups; *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with control group. Solvent without any substances was used as control group.

Accordingly to our results the genistein derivatives revealed a higher/equal antiproliferative activity taking as reference the native compound on both cancer cell lines. The cell viability of A431 cells was decreased by the native compound, genistein to 73%, whereas its esters induced a higher cytotoxicity 68% (GMAE), 66% (GPAE), and 65% (GSAE) (Figure 6). On A375 cells, the biocompounds exhibited similar values of cytotoxicity as the native compound, yet slightly smaller as follows: 81% for genistein and 79% (GMAE), 77.5% (GPAE) and 77% (GSAE), respectively (fig. 7). Previous studies [15-18] have also revealed a significant antitumor activity of genistein on several cancer cell lines; therefore their use as reference substances for the evaluation of the new compounds is completely justified.

Conclusions

We have synthesized and tested three genistein fatty acid esters on two tumor cell lines: A431 (skin epidermoid carcinoma) and A375 (human melanoma cells). Our

results showed that the new biocompounds exhibited a similar or even higher antiproliferative effect on tumor cells as compared to the native compound what makes this compounds promising molecules for further *in vivo* studies.

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