Effect of Free Radicals on the Biological Action of Genistein *In Vitro* and Synergism with Mitomycin C

JOHANNES HARTMANN and NIKOLA GETOFF

Section of Radiation Biology, Department of Nutritional Sciences, The University of Vienna, Vienna, Austria

Abstract. *Background: The effect of oxidizing and reducing free radicals on the biological properties of genistein (GEN) were investigated in vitro. Materials and Methods: MCF-7 cells were treated with GEN (5 μmol or 40 μmol) and/or mitomycin C (MMC; 2.5 μmol). For testing a possible radiosensitizing and synergistic effect of GEN in relation to MMC, a radiation dose of γ-rays was applied up to 8 Gy. In order to produce the desired free radicals, the media were saturated with: (i) air (46% OH and 54% $O_2^{\bullet-}$), (ii) N_2O (90% OH, 10% H), or (iii) argon (44% e^-_{aq}, 10% H and 46% OH). The survival fractions of the cells were determined by clonogenic assay. Results and Conclusion: GEN was found to be able to sensitize MCF-7 cells against the action of reducing (e^-_{aq}, H, R$^{\bullet}$) as well as of oxidizing (OH, $O_2^{\bullet-}$) free radicals. This ability was enhanced with increasing GEN concentration. Moreover, GEN acted synergistically with MMC in reducing as well as in oxidizing environments.*

Genistein (GEN; 4′,5,7 trihydroxyisoflavone) is a phyto-estrogen of the isoflavonoid group. It is contained mainly in soya beans in addition to genistin and daidzin. Based on its molecular structure (Figure 1), it offers several positions for free radical attacks, leading to formation of several transients, which may have various biological properties. This fact has initiated an abundant number of studies in the last two decades, covering a broad spectrum of objectives. It was established for example that genistein is involved in various processes: the reduction of oxidative DNA damage and lipid peroxidation (1), regulation of HMG-CoA-reductase in MCF-7 human breast cancer cells (2), increase of the radiation effect in cervical cancer cells (3), inhibition of the invasive potential of human hepatocellular carcinoma by altering the cell cycle (4), and the action of the synthetic isoflavone, phenoxodiol, in the management of ovarian and other forms of human cancer (5).

The anticancer therapeutic potential of soya isoflavones has been discussed in a review paper (6). It has also been established that genistein is a very good scavenger for strongly reducing e^-_{aq} [k (e^-_{aq} + GEN)=6.2×10^9 $lmol^{-1}$ s^{-1}] (7) as well as for oxidizing OH-radicals [k (OH + GEN)=2.3×10^{10} $lmol^{-1}$ s^{-1}] (8). This fact classifies genistein as a substance with many biological properties.

Oxidizing (*e.g.* OH, $O_2^{\bullet-}$) as well as reducing (*e.g.* e^-_{aq}, R•) free radicals are generated in the human body. They play a determining role in the correct course of the involved biological processes. The same free radicals can also be produced by ionizing radiation in aqueous media. Following this pathway, one has the possibility for investigating their specific action on selected substrates under desired experimental conditions by experiments *in vitro* or *in vivo*.

It should also be pointed out that biological compounds having functional groups such as –OH, $–NH_2$, $–NHCH_3$, $–HPO_4^{2-}$ and –SH can eject electrons from their singlet excited states (9). This process is in competition with the fluorescence of a given compound. Since GEN possesses three –OH groups, it appears to act as a potent electron donor in addition to its other biological abilities. Very recently, it was reported that the use of genistein sensitizes the radiation effect on lung cancer cells. This finding contributes to a better therapy efficiency (10).

Taking all these properties into account, the aim of the present work embraces: (i) comparison of the antitumor effect of genistein (GEN) in various aqueous media, where oxidizing or reducing free radicals, or mixture of both types are involved; (ii) investigations of a possible synergistic effect of GEN on mitomycin C (MMC) used as a typical cytostatic agent.

Materials and Methods

Chemicals. The applied chemicals were of the highest purity available (Fluka-Aldrich, Merck). Mitomycin C (MMC; Kyowa Hakko Kogyo Co Ltd, Tokyo, Japan) was used as obtained. The media and the supplements for the cells were obtained from

Correspondence to: Professor Nikola Getoff, Section of Radiation Biology, Department of Nutrition Science, The University of Vienna, UZA II, Althanstr. 14, A-1090 Vienna, Austria. Tel: +43 1 427754966, Fax: +43 1427754965, e-mail: nikola.getoff@ univie.ac.at

Key Words: Genistein, phytoestrogen, free radical, synergism, mitomycin C.

Invitrogen GmbH (Lofer, Germany). The aqueous solutions were prepared with triple distilled water and were saturated with high purity air, N_2O or argon, respectively in order to achieve the desired type of free radical under gamma irradiation.

Radiation. A Gammacell 220 (Nordion International Inc, Canada) instrument, providing a dose rate of 40 Gy min^{-1}[(a)], served as an irradiation source for the production of free radicals. The dose rate was controlled periodically by means of a modified Fricke-Dosimeter, using G (Fe^{3+})=15.6 (11), where G=number of formed or decomposed species on absorption of 100 eV energy.

Water radiolysis and yield of primary products. The water radiolysis (Eq. [1]) and the yield (G value)[(b)] of the primary active species, formed in the absence and in the presence of air as well as in media saturated with N_2O (conversion of e^-_{aq} into OH), are briefly discussed.

$H_2O \quad -\wedge\vee\wedge\vee\rightarrow e^-_{aq}, H, OH, H_2, H_2O_2, H^+_{aq}, OH^-_{aq}$ Eq. [1]

with at pH G values ~6.5 to 8.5: (2.7) (0.6) (2.8) (0.45) (0.72) (3.2) (0.5), respectively.

Total radical G (e^-_{aq} + H + OH)=6.1=0.612 μmol J^{-1}=100% Eq. [2]

The above G values are valid for air-free media. In the presence of air, e^-_{aq} and H are converted into peroxyl radicals, hence the reactive species are: 46% OH and 54% $O_2^{\bullet-}$:

$H + O_2 \rightarrow HO_2^{\bullet}$ ($k=2\times10^{10}$ $lmol^{-1}$ s^{-1}) Eq. [3]

$e^-_{aq} + O_2 \rightarrow O_2^{\bullet-}$ ($k=1.9\times10^{10}$ $lmol^{-1}$ s^{-1}) Eq. [4]

$HO_2^{\bullet} \rightarrow H+ + O_2^{\bullet-}$ (pK=4.8) Eq. [5]

In aqueous solutions saturated with N_2O, e^-_{aq} is specifically converted into OH radical:

$e^-_{aq} + N_2O \rightarrow OH^+ + OH^- + N_2$ ($k=0.9\times10^{10}$ $lmol^{-1}$ s^{-1}) Eq. [6]

In such cases, 90% OH and 10% H are involved in the initiated processes.

Cell culture. As a model for the experiments *in vitro*, human breast adenocarcinoma MCF-7 cells were used. The cells were maintained in a humidified atmosphere containing 5% CO_2 using a Cytoperm incubator (Heraeus, Vienna, Austria). The cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% glutamine and 0.5% penicillin/streptomycin.

Clonogenic assay. Logarithmically growing cells (1×10^5 cells/dish) were put into sterilized plates (diameter 10 cm) and allowed to attach overnight. Media were then changed and the cells were incubated for 48 h with different concentrations of GEN (5 μmol, 40 μmol), mitomycin (MMC; 2.5 μmol), or in a mixture of both (40 μmol GEN + 2.5 μmol MMC). After 48 h, the samples were irradiated with γ-rays (12, 13). Samples were stained with 0.5% crystal violet solution for 15 min and colonies (>50 cells) were scored as survivors. Each experiment was performed in triplicate and repeated at least three times.

Statistical calculation. The calculated standard deviation of several determinations were found to be less than 5% .

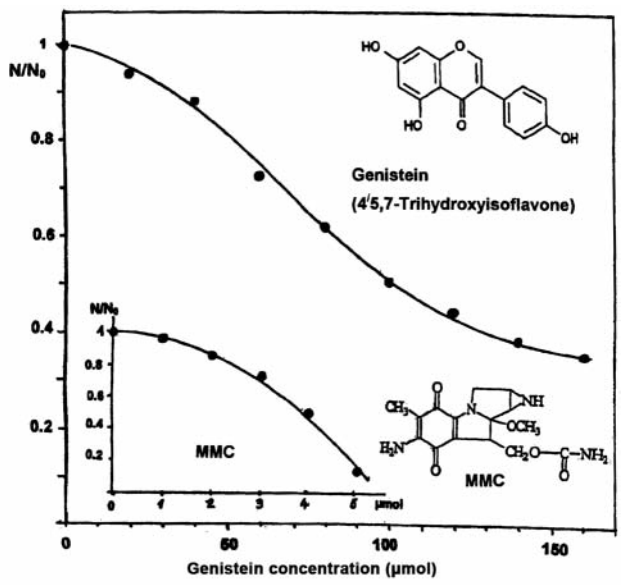

Figure 1. *Toxicity (N/N0 ratio) as a function of genistein concentration; Model: Breast cancer cells (MCF-7) in DMEM (pH ~7.4) in the presence of air. Inset: MMC toxicity on MCF-7 cells under the same conditions.*

Results and Discussion

Toxicity of GEN and MMC. The toxicity of both GEN and MMC in MCF-7 cancer cells was investigated under the same conditions in aerated aqueous media (pH ~7.4; incubation time 48 h for comparison). The obtained results are presented in Figure 1 as N/N_0 ratio in dependence of the applied substrate concentration, where: N_0 = number of cells before treatment and N afterwards. From the observed N/N_0 ratio, it is obvious that toxicity of MMC is much stronger than that of GEN. Based on these data, appropriate concentrations of GEN and MMC were implemented in the *in vitro* experiments.

Effect of oxidizing radicals on GEN and MMC activity. Action of OH and $O_2^{\bullet-}$ species. First, the survival curves (N/N0 – ratio of MCF-7 cells) were studied in dependence of the acting radical concentration (proportional to the absorbed radiation dose, Gy) in aerated aqueous media (pH ~7.4). In order to get a deeper insight in the involved process, the course of the curves was followed for GEN and MMC individually as well as in mixtures of both. Under the applied experimental conditions only oxidizing radicals (46% OH, 54% $O_2^{\bullet-}$) are operating.

[(a)]1Gy (Gray)=100 rad=6.24×10^{15} eV/g absorbed energy; [(b)]G value=number of changed molecules per 100 eV (1.60×10^{17}J) absorbed energy.
For conversion into SI units: multiply the G value by 0.10364 to obtain G (×) in μmol/J.

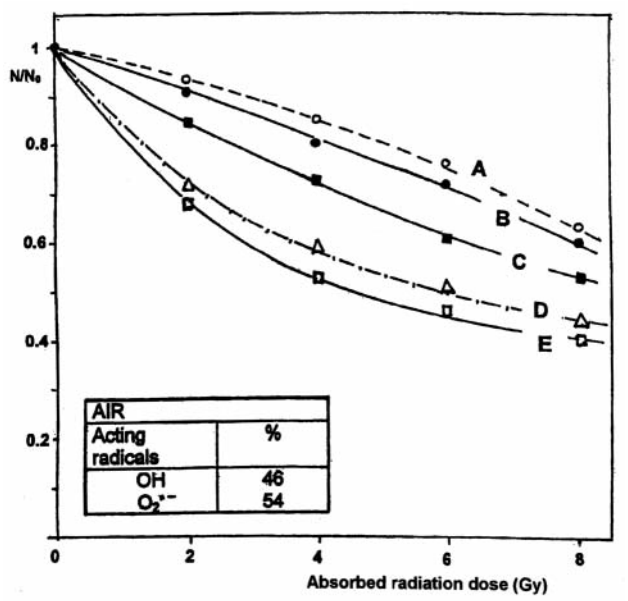

Figure 2. *Survival curves (N/N_0 ratio) as a function of absorbed radiation dose (Gy) of MCF-7 cancer cells in aerated aqueous solutions (pH ~7.4) of various systems: (A) buffer, (B) 5 μmol GEN, (C) 40 μmol GEN, (D) 2.5 μmol MMC and (E) 40 μmol GEN + 2.5 μmol MMC.*

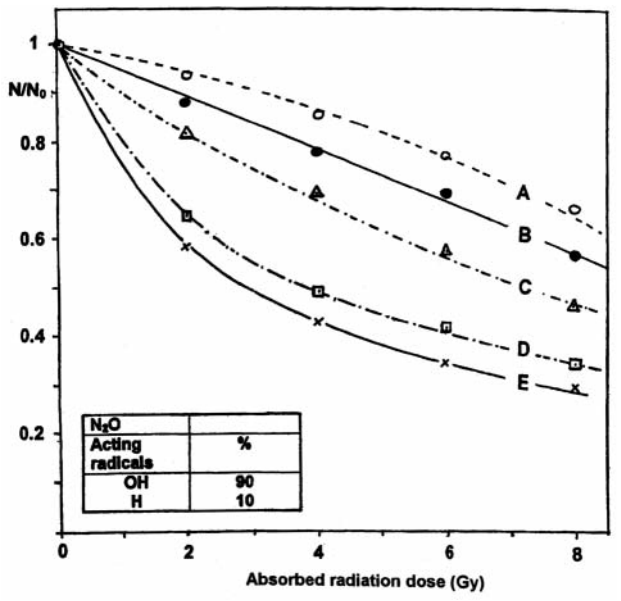

Figure 3. *Survival curves (N/N_0 ratio) as a function of absorbed radiation dose (Gy) for MCF-7 cancer cells in aqueous media (pH ~7.4) saturated with N_2O: (A) buffer, (B) 5 μmol GEN, (C) 40 μmol GEN, (D) 2.5 μmol MMC and (E) 40 μmol GEN + 2.5 μmol MMC.*

As expected, the cytostatic effect of MMC is much stronger then that of GEN (compare curves B and C with D in Figure 2). Furthermore, it is clearly demonstrated that increasing the GEN concentration from 5 μmol (curve B) to 40 μmol GEN (curve C, Figure 2), the antitumor activity of GEN is correspondingly enhanced. It is also interesting that GEN acts synergistically with the cytostatic action of MMC (curve E). This effect indicates an electron transfer process from GEN to MMC, as previously observed for other substrates (12-15). In this connection, it should be mentioned that not only the semiquinone of MMC ($MMC^{\bullet-}$), but, in the presence of air, also the oxidized forms of MMC, such as OH adducts (MMC.OH) and peroxyl radicals ($MMC.O_2^{\bullet}$) (16) as well as radical cation ($MMC^{\bullet+}$) (17) were involved in the cytostatic action of MMC. The reaction mechanism of the system in aerated media appears to be rather complicated. This is demonstrated by the reaction probability (P) of both MMC and oxygen, where P=product of reaction rate constant (k) and substrate concentration (mol/l). It is obvious that a competition for e^- between MMC and O_2 occurs, since: k (MMC + e^-_{aq}) = 2.2×10^{10} $lmol^{-1}$ s^{-1} (13) and k (O_2 + e^-_{aq})=1.9×10^{10} $lmol^{-1}$ s^{-1} (Eq. 4) in addition to the electron transfer from $MMC^{\bullet-}$ to O_2 with a rate constant rate: k ($MMC^{\bullet-}$ + O_2 → MMC + $O_2^{\bullet-}$)=2×10^9 $lmol^{-1}$ s^{-1} (18). Based on all these facts, it is clear that several parameters are implicated in the process, which can affect the final results. The OH radicals can attack GEN predominantly at double bonds at various positions (formation of OH adducts) as well as scavenge e^-_{aq} released by GEN. The above facts demonstrate the complexity of the involved processes.

Action of OH and H species. In aqueous media saturated with N_2O, the radiation-induced processes are initiated by 90% OH (strongly oxidizing) and 10% H (strongly reducing) free radicals. The mean values of the survival curves of these experiments, under otherwise the same conditions as before, are depicted in Figure 3. The survival curves, N/N0 ratio in dependence of the absorbed radiation dose, are similar to those shown in Figure 2, however the observed effects are much more strongly expressed. This fact suggests that the OH radicals are decisive in regard to reactivity towards GEN, k (OH + GEN)=2.3×10^{10} $lmol^{-1}$ s^{-1} [10] and MMC, k (OH + MMC)=5.8×10^8 $lmol^{-1}$ s^{-1} (15). In this case, the antitumor action of the MMC transients, as well as very likely the involvement of the final products resulting from OH attack on MMC, are again demonstrated. The synergistic effect of GEN with MMC (curve D and E, Figure 3) is likewise more strongly pronounced, as in aerated media (Figure 2).

Action of reducing (e^-_{aq}, H) and oxidizing (OH) free radicals on GEN and MMC activity. Experiments *in vitro* using MCF-7 cancer cells in aqueous media (pH ~7.4), saturated with argon, containing GEN or MMC alone as well as mixture of both, were performed. In this case, reducing (44% e^-_{aq} and 10% H) as well as oxidizing (46% OH) both

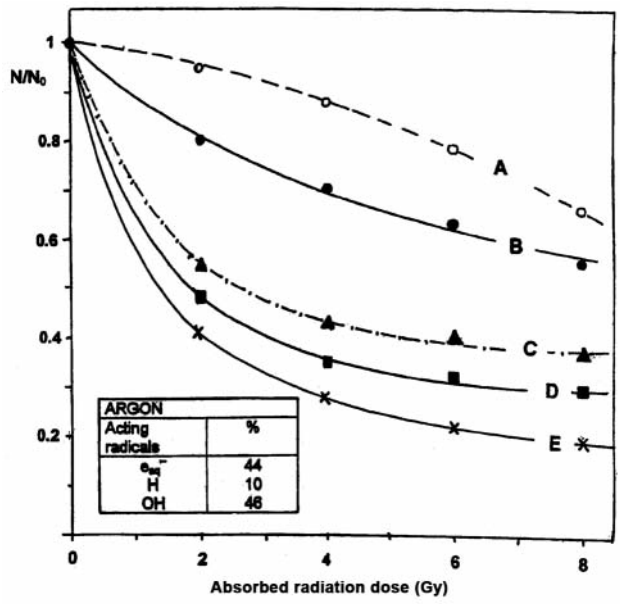

Figure 4. *Survival curves (N/N_0 ratio) as a function of absorbed radiation dose (Gy) for MCF-7 cancer cells in air-free aqueous solutions (pH ~7.4) in various media: (A) buffer, (B) 5 μmol GEN, (C) 40 μmol GEN, (D) 2.5 μmol MMC and (E) 40 μmol GEN + 2.5 μmol MMC.*

free radicals in about equal concentration are operating. The mean values obtained in several series of experiments are presented in Figure 4.

Under the present experimental conditions, the course of all survival curves expresses much stronger antitumor effect in comparison to those shown in Figures 2 and 3. Obviously, the GEN as well as MMC transients formed by the attack of reducing species (e^-_{aq}, H), namely $GEN^{\bullet-}$ and $MMC^{\bullet-}$, have much stronger antitumor activity. Naturally, the GEN and MMC transients, produced by the corresponding OH attack, also contribute to the antitumor effect.

Here, again, with increasing the GEN concentration, antitumor efficiency is also enhanced (see curves B and C, Figure 4). In addition to this, the synergistic effect of GEN with respect to MMC is also highly expressed (compare curves D and E, Figure 4).

The role of final products. Commonly, less attention is given to the biological effects of the products originating from the free radical attack on a given substrate. This subject matter is of special interest because the biological influence of these products is always implemented in the results of the experiments *in vitro*. The final products of the substrate may also be responsible for the frequently observed, undesired side-effects. Following these aspects, the GEN degradation by free radicals and the formation of final products under various conditions has been separately reported (19). The highest decomposition effect was observed in the presence of air. As final products of GEN in aerated media, a mixture of aldehydes and carboxylic acids was determined, in addition to as yet unidentified products.

Conclusion

The highlights of the experiments of the present work can be stressed as follows. In aerated media (Figure 2, acting radicals: 46% OH and 54% $O_2^{\bullet-}$), GEN exhibits a strong antitumor effect, which rises with increasing concentration. Additionally, GEN shows a synergistic effect on MMC antitumor action, very likely due to an electron transfer process. In solutions saturated with N_2O (Figure 3, acting species: 90% OH, 10% H), practically the same effect was observed as in aerated media; note particulary, the nexus between MMC and the mixture of MMC and GEN (Figure 3, curves D and E). In air-free media (Figure 4, acting radicals: 44% e^-_{aq}, 10% H and 46% OH) both effects, *i.e.* the antitumor action of GEN as well as its synergistic action on MMC, are much more strongly expressed, certifying the role of solvated electrons (e^-_{aq}).

Summing up, it can be stated that GEN shows an antitumor property and can increase the efficiency of MMC in reducing as well as in oxidizing environments. The concentration of the substrates play a decisive role.

Acknowledgements

The authors are indebted to Greiner Holding GmbH, KG & AG, Kremsmünster, Austria, for placing at our disposal radiation-sterilized utensils for the *in vitro* experiments. Special thanks are also expressed to Dr. Böhm® (APOMEDICA GmbH, Graz, Austria) for making fine chemicals available.

References

1 Cai Q, Rahn OR and Zhang R: Dietary flavonoids, quercetin, luteolin and genistein, reduce oxidative DNA damage and lipid peroxidation and quench free radicals. Cancer Lett *119*: 99-107, 1997.

2 Duncan RE, El-Sohemy A and Archer MC: Regulation of HMG-Co-reductase in MCF-7 cells by genistein, EPA and DHA, alone and in combination with mevastatin. Cancer Lett *224*: 221-228, 2005.

3 Yashar CM, Spanos WJ, Taylor DD and Gercel-Taylor C: Potentiation of radiation effect with genistein in cervical cancer cells. Gynecol Oncol *99*: 199-205, 2005.

4 Gu Y, Zhu CF, Iwamoto H and Chen JS: Genistein inhibits invasive potential of human hepatocellular carcinoma by altering cell cycle, apoptosis and angiogenesis. World J Gastroenterol *11*: 6512-6517, 2005.

5 Morre DJ, Chueh PJ, Yagiz K, Balicki A, Kim C and Morre DM: ECTO-NOX target for the anticancer isoflavone phenoxodiol. Oncology Res *16*: 299-312, 2007.

6 Ravindranath MH, Muthugounder S, Presser N and Viswanathan S: Anticancer therapeutic potential of soy isoflavone, genistein. Adv Exp Med Biol *546*: 121-165, 2004.

7 Zielonka J, Gebicki J and Grynkiewicz G: Radical scavenging properties of genistein. Free Radic Biol Med *35*: 958-965, 2003.
8 Cai Z, Li X and Katsumura Y: Interaction of hydrated electron with dietary flavonoids and phenolic acids: rate constants and transient spectra studied by pulse radiolysis. Free Radic Biol Med *27*: 822-829, 1999.
9 Getoff N: A review on the relationship between Q (e^-_{aq}) and Q (F) of excited compounds in aqueous solution. Radiat Phys Chem *34*: 711-719, 1989.
10 Hermann RM, Fest J, Christiansen H, Hille A, Rave-Fränk M, Nitsche M, Gründker C, Viereck V, Jarry H and Schmidberger H: Radiosensitization dependent on p53 function in bronchial carcinoma cells by the isoflavone genistein and estradiol *in vitro*.Strahlenther und Onkol *183*: 195-202, 2007.
11 Getoff N: Chemical Dosimetry. *In*: Radiation Chemistry. Fundamentals Techniques and Application (German). Kaindl K and Granl FH (eds.). A. Hüthig Publ. House, Heidelberg, Germany. pp. 226-249, 1967.
12 Bader Y and Getoff N: Effect of resveratrol and mixtures of resveratrol and mitomycin C on cancer cells under irradiation. Anticancer Res *26*: 4403-4408, 2006.
13 Hartmann J and Getoff N: Radiation-induced effect of adenine (vitamin B4) on mitomycin C activity. *In vitro* experiments. Anticancer Res *26*: 3005-3010, 2006.
14 Getoff N: Cytostatic efficiency enhancement by vitamins C, E and β-carotene under irradiation. State of the art. Radiat Phys Chem *60*: 351-358, 2001.
15 Delipetar-Grudl A and Getoff N: Radiation-induced sensitizing effect of folic acid (vitamin B11) and its synergistic action with mitomycin C: *in vitro* experiments. Oncology Res *14*: 559-566, 2004.
16 Getoff N, Solar S and Quint RM: One electron oxidation of mitomycin C and its corresponding peroxyl radicals. A steady-state and pulse radiolysis study. Radiat Phys Chem *50*: 579-583, 1997.
17 Getoff N: Mitomycin C radical cation studied by pulse radiolysis. In Vivo *17*: 173-176, 2003.
18 Getoff N: Effect of antioxidants and oxygen in radiotherapy. *In*: Progress in Radio-Oncology VII. (eds.). Kogelnik HD, Lukas P and Sedelmayer F. Proc 7th. Int Meeting On Progress in Radio-Oncology, Salzburg, Austria, May 15-19 2002. Monduzzi Editores, Bologna, Italy, pp. 99-105, 2002.
19 Hartmann J, Quint RM and Getoff N: Products resulting from free radical attack on aqueous genistein. Radiat Phys Chem *77*: 612-616, 2008.

Received January 29, 2008
Revised April 7, 2008
Accepted May 22, 2008