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# *In vitro* Apoptotic Bioactivity of Flavonoids from *Astragalus verrucosus* Moris

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Six aglycone flavonoids and their corresponding glycosides: genistein and genistin, quercetin and rutin, apigenin and apigenin 7-*O*-β-D-(6-*p*-coumaroyl) glucoside, as well as the aglycone daidzein isolated from *Astragalus verrucosus* Moris, were tested for their apoptosis-inducing potential. *In vitro* techniques that monitor bioactivity through morphological and biochemical changes were carried out on HCT116 (human colon carcinoma) and MCF7 (human Caucasian breast adenocarcinoma) cancer cell lines. Dose-dependent cytotoxic effects were monitored through changes in mitochondrial dehydrogenase activity using the standard MTT assay. The median inhibitory concentration ($GI_{50}$) determined from the dose-response curves showed that the aglycones apigenin and quercetin were the most bioactive (low $GI_{50}$), whilst daidzein and genistein, which had not been previously tested on these cell lines, showed a smaller cytotoxic effect (high $GI_{50}$). The remaining flavonoids, mostly glycosides, showed negligible cytotoxicity. Morphological changes were monitored by microscopic observation with a photographic record. Results showed important hallmarks of apoptosis, including cell rounding with reduction of cell volume, small condensed nuclei, membrane blebbing and formation of apoptotic bodies.

**Keywords:** Flavonoids, aglycones, glycosides, apoptosis, cancer cell lines, HCT116, MCF7.

*Astragalus verrucosus* Moris, a very rare perennial endemic plant belonging to the family Fabaceae (Leguminosae), grows in a restricted area of south-western Sardinia, Italy [1]. The genus *Astragalus* is well known in Chinese folk medicine because its properties are somewhat similar to those of the more expensive herb ginseng (*Panax ginseng*) and have been used as a substitute for this species [2]. The roots of various *Astragalus* species have been used to increase body resistance against viral infections, to re-balance the immune system and for their tonic action on the liver. Extracts from *Astragalus* species have been used as antiperspirant, diuretic and general tonic agents. The roots have been applied in the treatment of diabetes mellitus, nephritis, and bacterial infection, as well as against leukaemia and uterine cancer [3,4]. Phytochemical studies of *A. verrucosus* have shown the presence of several classes of flavonoids, such as flavonols, isoflavones, and flavones, both as aglycones and glycosides (Table 1). Over 6000 different flavonoids (aglycones) have been identified to date, and this figure increases considerably if their corresponding glycosides are included [6-8]. Flavonoids are plant secondary metabolites, especially widespread in the plant kingdom and ubiquitous in photosynthesising cells. Flavonoids represent important dietary constituents because of their antioxidant activity. They are accumulated in different plant parts including fruits, vegetables, nuts, seeds, and flowers, as well as in products derived from them, such as propolis, honey and tea [5].

Flavonoids are well known for their different pharmacological properties, including: anti-allergic, immunoregulatory, antioxidant, anti-inflammatory, hypotensive, antibacterial, antifungal, antiviral, cytotoxic and osteogenic activities [6-10]. Preparations containing these compounds have been used to treat different diseases for centuries. Some are borrowed directly from nature, as for example propolis, which has been used since antiquity to heal

**Table 1**: Typical flavonoids of *Astragalus verrucosus*

| FLAVONES | REF. |
|---|---|
| apigenin | [19] |
| apigenin 7-*O*-β-D(6"-*p*-coumaroyl)glucoside | [19] |
| **FLAVONOLS** | |
| kaempferol 3-*O*-rutinoside | [19] |
| kaempferol 3-robinobioside | [24] |
| rutin | [15] |
| quercetin | [4] |
| **ISOFLAVONES** | |
| daidzein | [19] |
| daidzin | [19] |
| ononin | [25] |
| calycosin | [17] |
| pseudobaptigenin | [15] |
| genistein | [19] |
| genistin | [19] |
| pratensein | [19] |

sores and wounds. Several flavonoid-rich foods (such as quercetin-rich apples) have been reported to lower the risk of different types of cancer, including hepatoma, lung and breast cancer [8]. Flavonoids such as quercetin have been reported to inhibit the growth of various cell lines derived from human cancers [6,9]. They have been shown to be selective cytotoxic agents to a range of cancer cell lines, including Jurkat, PC-3, colon 205 and HepG2. The mode of cell death in the human promyelocytic leukaemia HL-60 cell line treated with the structurally related flavonoids apigenin, quercetin, myricetin, and kaempferol has been established as being apoptotic through the release of cytochrome C, as well as caspase-9 and caspase-3 into the cytosol [7,11]. Other flavonoids have been studied and shown to be capable of inducing cell death by apoptosis [12]. Interestingly, quercetin was found to restore apoptosis sensitivity in cell lines usually resistant to apoptosis [13]. Terminal differentiation has also been identified as another possible mechanism of flavonoid action and, therefore, these compounds can serve as potential chemotherapeutic agents [14]. Most plant-derived natural products are often produced as a mixture of related compounds that have a synergistic mode of action, either among themselves or with other compounds, and flavonoids are no exception. The synergistic effects of three isoflavones: genistein, biochanin-A and daidzein have also been demonstrated in cancer cell lines through cell growth inhibition, cell cycle changes, and induction of apoptosis. Enhanced expression of pro-apoptotic caspase 3 and downregulation of anti-apoptotic Bcl-2 was also demonstrated [8,11,14]. Of increasing interest also is the potential role of flavonoids in reducing the problems of drug resistance to antimicrobials and in cancer therapy. This has also been extensively investigated and holds some promise, especially with the increasing problems associated with β-lactamase resistance and MRSA [6,7].

This current work was addressed to test the bioactive potential and apoptosis-inducing activity of some characteristic flavonoids isolated from *A. verrucosus* on HCT-116 and MCF-7 tumor cell lines. These compounds include two aglycones (daidzein and genistein) that have not been previously tested on these cell lines. Although work on the induction of apoptosis in cancer cells by flavonoids appears to be gaining momentum, there is still insufficient published data on the action of the various flavonoids on different cell types. The NCI database gives the average $GI_{50}$ values for its 50 cancer cell line panel as 27 μM for apigenin and 59 μM for quercetin. Though the values for the individual cell lines are not available, these average values confirm the trend that we obtained for the same test compounds in this work. This research has also confirmed the trend in the published literature that the aglycone flavonoids are generally more potent than their corresponding glycosides. What seems to be omitted in the literature is a reference to the paradoxical increase in cell proliferation on exposure to low concentrations of the aglycones, something which has also been consistently observed with another group of natural compounds, the mono-sesqui- and di-terpenoids. Flavonoid molecules have structural similarities and comparable molecular sizes to the diterpenoids. Like diterpenoids, aglycone flavonoid molecules are planar and have been shown capable of interacting, to various degrees, with the phospholipid bilayer. The degree of flavonoid interaction has been shown to vary according to whether hydroxyl groups are present or absent, the number of hydroxyl groups and the position of their attachment to the A and B ring [5,15]. Apart from the chemical interactions of the side groups, the small size of the aglycones allows for a greater chance of interacting deep within the inner layers of the phospholipid bilayer and brings about changes in membrane fluidity. Such changes in membrane fluidity as a result of flavonoid interactions have been reported both for prokaryotic and eukaryotic cells, as a result of which cells lose important capabilities. For instance, flavonoid action on the inner membrane of Gram-positive bacteria has been reported to lead to dissipation of membrane potential electrochemical gradient with consequent reduction of ATP synthesis, membrane transport and motility. In other experiments, the action of flavonoids leads

to increased permeability of the inner membrane with loss of important cell constituents such as $K^+$ ions [5]. A similar effect is reported for a number of flavonoids, including quercetin; this has been shown to interact with the membrane of the mitochondria, decreasing its fluidity and, as a result, either inhibiting the respiratory chain or inducing membrane permeability transition [15]. The latter is equivalent to a state where the mitochondria lose their function and release apoptosis-inducing factors that lead to cell death by apoptosis.

The aglycone flavonoids that have been shown to be the most bioactive represent relatively small molecules that can easily interact with the cell membrane. Conversely, since the glycoside flavonoids represent a bulkier molecule, of which only a part can interact with the phospholipid bilayers, they are unable to interact deeply enough inside the membrane to cause drastic changes in fluidity. This hypothesis could neatly explain the differences observed in the action of different aglycone and glycoside flavonoids where for the glycosides, a relatively flat dose-response curve was maintained, even at high concentrations, indicating that interaction does not increase, even under an appreciable concentration gradient.

The relevance of the role of aglycone flavonoids in the induction of apoptosis in cancer cells has to be considered in the light of emerging research as to the role of mitochondria in cancer. Formerly**,** it was thought that cancer cells are predominantly glycolytic because their mitochondria were defective and incapable of generating the vast quantities of ATP needed to sustain growth of cancer cells. However**,** the evidence points to fully functional mitochondria that are pushed into an inactive state so as to suppress the apoptotic cascades that are normally initiated in mitochondria [16,17]. Thus**,** this perturbation of membranes by natural products such as flavonoids could explain why apoptosis results when cancer cells are treated with these compounds. A change in membrane fluidity (especially of the mitochondrial membrane) could lead to the release of pro-apoptotic signals from the mitochondria, such as cytochrome c, and trigger the apoptotic cascade which eventually results in cell death.

## Experimental

***Extraction, flavonoid isolation and identification:*** *Astragalus verrucosus* dried aerial parts were extracted in a Soxhlet apparatus using different solvents with increasing polarity (*n*-hexane, chloroform, and methanol) [4]. The methanolic extract was purified by gel permeation (Sephadex LH-20, MeOH-Water 8:2) and medium pressure liquid chromatographic steps ($SiO_2$ RP-9, MeOH-water 7:3). Seven flavonoids: apigenin, apigenin 7-*O*-β-D-(6*p*-coumaroyl) glucoside, quercetin, rutin, daidzein, genistein and genistin were isolated and identified by NMR and MS experiments, and by comparison with authentic samples and literature data [4,18,19]. Table 1 gives additional data on other flavonoids isolated from *A. verrucosus* [21,22].

***Cell lines and medium***: Two adherent cell lines, namely human colon carcinoma cell line HCT 116 and human Caucasian breast adenocarcinoma cell line MCF 7, were obtained from ECACC Porton Down, Salisbury, UK. RPMI-1640 medium with 2 mM L-glutamine and 1mM sodium pyruvate (Gibco BRL, Life Technologies) was supplemented with 10% fetal bovine serum (Gibco) and 25 IU/mL penicillin G and 25 µL/mL streptomycin (PenStrep, Gibco). All cell lines were kept in exponential growth phase by twice weekly subculture in $T_{25}$ cell culture flasks (Nunc, Kampstrum, Denmark) in 6 mL of medium using a split ratio of 1:5.

**Table 2**: Results of apoptotic activity in HCT116 and MCF7 cancer cell lines for the flavonoids isolated from the methanolic extract of *Astragalus verrucosus*. All median inhibitory concentration values ($GI_{50}$) shown are an average of at least three replicates.

| | HCT116 ($GI_{50}$) | | | | | MCF7 ($GI_{50}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **COMPOUNDS** | **replicate** | **1** | **2** | **3** | **Average (µg/mL)** | **replicate** | **1** | **2** | **3** | **Average (µg/mL)** |
| **Quercitin** | | 9.0 | 20.9 | 28.8 | 19.6 | | >100 | 47.2 | 9.8 | 28.5 (>100) |
| **Daidzein** | | 76.8 | >100 | >100 | 76.8 (>100) | | 66.2 | >100 | >100 | 66.2 (>100) |
| **Apigenin** | | 5.9 | 3.3 | 2.8 | 4.0 | | 5.7 | 4.2 | 4.0 | 4.6 |
| **Genistein** | | 33.2 | >100 | 53.9 | 43.5 (>100) | | 60.2 | 37.8 | 64.9 | 54.3 |
| **Rutin** | | >100 | >100 | >100 | >100 | | >100 | >100 | >100 | >100 |
| **apigenin -7-*O*-β-D-(6''-p-coumaroyl)-glucoside** | | >100 | >100 | >100 | >100 | | >100 | >100 | >100 | >100 |
| **genistin** | | >100 | >100 | >100 | >100 | | >100 | >100 | >100 | >100 |

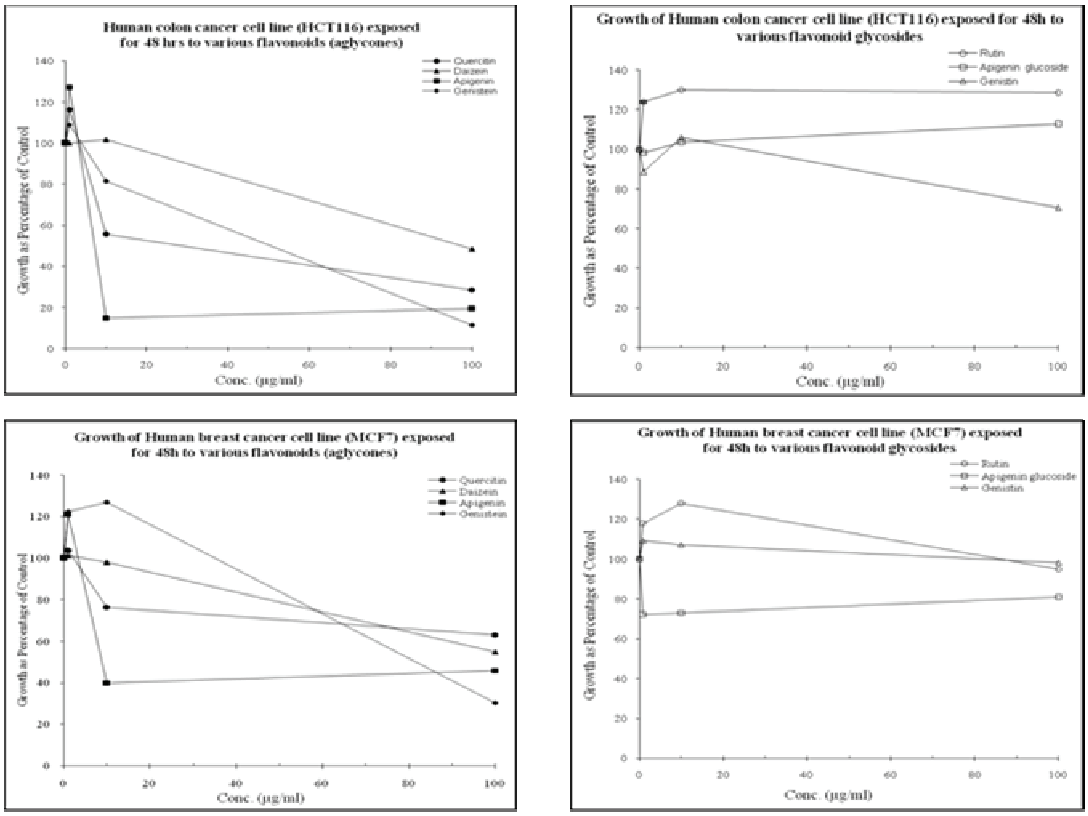


**Figure 1**: Dose-response curves for 48 h exposure of HCT116 (top) and MCF7 (bottom) cancer cell lines treated with various concentrations of aglycone flavonoids (left) and their corresponding glycosides (right). All values are plotted as a percentage of control absorbance values. Absorbance values were measured at 550 nm using the standard MTT photometric assay.

***MTT assay***: Cell cultures in exponential growth phase were trypsinised with 0.25% trypsin in EDTA, and after a viable cell count diluted to give a seeding density of 5000 cells per 180 µL of culture medium. The cells were then plated in flat-bottomed 96-well micro titer plates (Nunc) and incubated overnight at 37°C in a 5% $CO_2$ humidified atmosphere. A time zero ($T_0$) reference plate was also set up to check cell viability at the point of first drug exposure. The appropriate dilutions of the flavonoids in culture medium were prepared from a stock solution of 40µg/µL in DMSO and 20µL aliquots of the drug were added per well to give a final concentration range from 0.01 µg/mL to 100 µg/mL, plus solvent controls. Plates were further incubated for a maximum of 44 h, with inspection at regular intervals to check growth progress. Cell viability was determined by the addition of a 50 µL aliquot of MTT (2.0 mg/mL) per well, and insoluble formazan crystals allowed to develop by incubating for a further 4 h. Formazan was dissolved by adding 125 mL of solubilization Sorensen's buffer (DMSO : glycine 4:1) to each well and the plates were agitated for 5 minutes. The absorbances were read using a microplate reader (BioTech $EL_x$ 808) at 550 nm and 650 nm wavelength. Dose-response curves were generated using a Delta soft 3 software package and in combination with the $T_0$ value, the median growth inhibition ($GI_{50}$) was determined for three independent trials. Absorbance values derived from the MTT assays were used to generate dose-response curves, from which the median inhibitory concentration ($GI_{50}$) was calculated. The average $GI_{50}$ values (µg/mL) for three independent trials are shown in Table 2. The cell growth was also plotted as a percentage of the control, and typical graphs are shown in Figure 1.

***Study of morphological changes***: These were restricted to a photographic record of the changes that were observed in the overall cell structure when visualised under an inverted microscope at high magnification. Photos were taken after 24 and 44 h exposure before adding the MTT. The dose-response curves for the aglycone flavonoids were typical of cytotoxic drugs with $GI_{50}$ values in the range of 4-76 µg/mL. The highest activity of the aglycones was for apigenin. Furthermore, its $GI_{50}$ values are very similar in the two cell lines tested with an average $GI_{50}$ for HCT116 of 4.0 µg/mL and

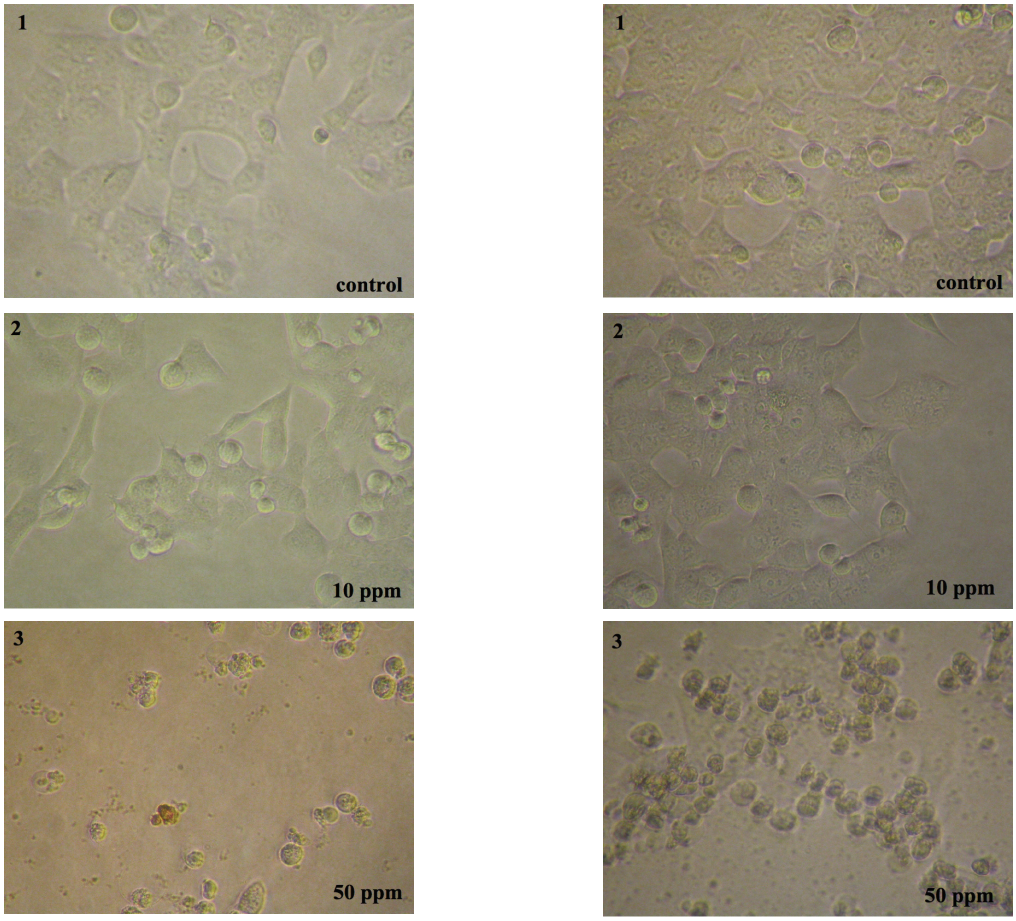


**Figure 2**: HCT 116 and MCF7 cells after 48 h treatment with quercitin (10 and 50 µg/mL) with solvent controls (top).

for MCF7 of 4.6 µg/mL. The bioactivity of quercetin was the next best with an average $GI_{50}$ value in the 20 µg/mL range for HCT116 and 28.5 µg/mL for MCF7. The largest $GI_{50}$ values for the aglycones and, therefore, the least bioactive were for genistein and daidzein. Conversely, the dose-response curves of their corresponding glycosides were in most cases rather flat and showed large $GI_{50}$s in excess of 100 µg/mL, indicating that there is little cytotoxic effect on these two cell lines. The poor bioactivity of the glycoside derivatives was also corroborated from the morphological observations. One interesting feature of the dose-response curves of some of the aglycones and glycoside flavonoids was that at very low concentrations, a paradoxical stimulation of growth occurred, sometimes reaching 130% of the control. This is usually followed by a steep decrease in activity as the concentration increases. This paradoxical increase in cell activity for very low concentrations has been reported for other natural products including mono- and diterpenoids and may be related to their as yet unexplained mode of action [23].

Morphological changes resulting from the exposure of cells to different types of flavonoids and different time periods demonstrated that the cells undergo apoptosis with increasing concentration and with increased exposure time. Figure 2 shows the two cell lines treated at the two concentrations (10 and 50 µg/mL) with solvent controls after 48 h exposure, although the full exposure included a concentration range from 1-100 µg/mL. These morphological changes were clearly demonstrated under the high power objective (x400) without the need of further staining since these changes were comparable to those obtained in previous works [23]. The major hallmarks were the loss of cellular extensions and substrate contact resulting in the formation of round, free floating cells, some with extensive membrane blebbing and formation of apoptotic bodies. A reduction in overall cell volume resulting from cytoskeletal disruption and the formation of a small pycnotic nucleus from DNA condensation were also noted. For the active flavonoids, changes such as partial loss of contact with substrate and rounding off were initially observed at 10 µg/mL after 24 hour

exposure, progressing to pronounced hallmarks of apoptosis, such as membrane blebbing and nuclear condensation as concentration increased. At 50 μg/mL the cells were completely spherical and lost all attachment to the substrate after the same exposure time.

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