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Antiproliferative activity of *Saponaria vaccaria* constituents and related compounds[§]

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ABSTRACT

Total methanolic extracts of *Saponaria vaccaria* seed derived from several varieties, as well as various purified components obtained through successive chromatographic separations of total extracts were evaluated for their growth inhibitory activity in WiDr (colon), MDA-MB-231 (breast), NCI-417 (lung) and PC-3 (prostate) human cancer cells as well as the non-tumorigenic fibroblast BJ (CRL-2522) cell line using MTT colorimetric assay. Purified bisdesmosidic saponins segetoside H and I were further examined using microscopy and apoptosis assays. Bisdesmosidic saponins exhibited dose-dependent growth inhibitory and selective apoptosis-inducing activity. Growth inhibitory effects were particularly strong in a breast (MDA-MB-231) and a prostate (PC-3) cancer cell line. Total extracts exhibited a different preference being most active against a colon cancer cell line (WiDr). In a comparison of varieties, all of the total seed extracts exhibited similar dose-dependent activities, but with some variation in potency. Monodesmosidic saponins vaccarosides A and B, phenolic vaccarin, and cyclopeptide segetalin A, co-occurring seed substituents, did not exhibit activity. The non-tumorigenic fibroblast cell line BJ (CRL 2522) was growth inhibited but did not undergo apoptosis when treated with bisdesmosidic saponins at low micromolar concentrations. Saponin-rich extracts from *Kochia scoparia* seed and *Chenopodium quinoa* were also evaluated alongside *Saponaria* saponins but did not exhibit activity. Closely related Quillaja saponins exhibited activity but were less potent.

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Keywords

Saponaria vaccaria, saponins, growth inhibition, apoptosis, cancer, MDA-MB-231, PC-3.

Abbreviations

IC_{50}, 50% inhibitory concentration; MS, mass spectrometry; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAD, photodiode array detection.

DISCLOSURES: A portion of this work was presented in Dr. Ramirez-Erosa's PhD thesis.

1. Introduction

The seed of the medicinal plant *Saponaria vaccaria* L.[1], is rich in flavonoids, cyclopeptides and significant amounts of mono- and bisdesmosidic triterpene saponins (see Fig. 1) [1,2]. It has been used in traditional Chinese medicine (Wang Bu Liu Xing) for promoting menstrual discharge and milk secretion, healing wounds, as a diuretic, astringent, and anti-cancer agent [3-5]. Saponin-rich plants have been used since ancient times for the treatment of a variety of ailments with saponins possessing structure-dependent biological and pharmacological properties at least partly responsible for the inherent activities, e.g. hemolytic, antifungal, anticholesterolemic, anti-inflammatory, immunostimulatory, anti-cancer, etc. [6-9]. Cyclopeptides and flavonoids, the other classes of compounds present in significant quantities in the seed, have also been noted in several medicinal species and also exhibit a wide range of structure-dependent bioactivities [10,11].

Seed extracts of this plant have shown potential anti-cancer activity. A screening study on the cytotoxic activity of aqueous extracts derived from seventy-one Chinese medicinal herbs conducted previously, indicated that the water extract of *S. vaccaria* inhibited the growth of a number of human and murine cancer cell lines (breast, lung, pancreas and prostate) *in vitro* [12,13]. More recently several purified saponins from *S. vaccaria* were reported to possess growth inhibitory activity against murine leukemia P388, human lung A549, and human prostate LNCaP cell lines *in vitro* [14]. Earlier, undefined fractionated seed extracts were observed to extend the life of tumor-implanted mice [15] and to reduce the volume of implanted tumors [16].

As much of the previously reported work was somewhat lacking in detail, it was decided to further investigate the antiproliferative activity of *Saponaria vaccaria* by comparing activity of seed extracts from different varieties, as well as other saponin-rich species, against a panel of human cancer cell lines, and to also further examine individual compounds found in the seed to ascertain the main sources of the reported antiproliferative properties and their possible mode of action.

2. Materials and methods

2.1 Plant material and seed extracts

[1] A.k.a. *Vaccaria segetalis, V. hispanica, V. pyramidata;* com. cow cockle, cow herb.

Different varieties of *S. vaccaria* seed were obtained for this study. *S. vaccaria* 'Scott WT' (wild type, SWT) was obtained from Eric Johnson, Agri-Food and Agriculture Canada, Scott Experimental Farm, Scott, Saskatchewan. Seed of *S. vaccaria* cv. 'Pink Beauty' (PB) and 'White Beauty' (WB) were obtained from CN Seeds Ltd, Pymoor, UK, and the Mongolia variety (MG) was obtained from the North Central Regional Plant Introduction Station, USDA-ARS, Ames, IA, USA (accession PI 597629 originating from Mongolia). Several rows of each seed variety were hand seeded in the University of Saskatchewan Horticulture plots in the summer of 2003 and 2004. Seeds were planted 1 to 2 cm. deep at a rate of 100 seeds per row (7 m). Plants were grown under dry land conditions except for initial watering to promote germination. Bulk seed was harvested in the fall. *Quillaja* saponin having a sapogenin content of 25% (ca. 100% saponin content) was purchased from Sigma (S4521), Oakville, Ont. Canada . *Quinoa* saponin was obtained from HeadsUp Plant Protectants Inc., Kamsack, SK, Canada. *Kochia* saponin was obtained from processing *Kochia scoparia* seed as described in ref. 17 Wang Bu liu Xing powdered seed was obtained from Botanicum Herbs, Box 329, Pembina, ND, USA.

2.2 Comparison of seed bioactivity from different Saponaria varieties

Ground seed (1 g) of each variety was mixed with 70% methanol (10 mL) and let stand overnight. The mixture was centrifuged and the pellet washed with a further portion of 70% methanol. The combined methanolic extract was concentrated *in vacuo* to dryness and the residues each dissolved in DMSO to afford a stock solution which was diluted to afford desired saponin concentrations for testing. 1 g of seed was assumed to contain 20 mg of saponin. The highest concentration in the assays was 50 µg/mL saponin equivalent. MS profiles of the different varieties are shown in Supplementary data 1.

2.3 Preparative Extraction and Fractionation

Seed of Pink Beauty variety was cleaned by screening to remove foreign seeds and a stream of air was used to remove debris as necessary. 100 g of cleaned seed was ground portion wise in a coffee grinder. Ground seed was mixed with 70% methanol (ca 600 mL) and let stand with occasional stirring for 1 day or more. The mixture was filtered through a large sintered glass funnel with minimal or no vacuum (or alternatively centrifuged at low speed to separate solids). The filter

cake was washed with warm 70% methanol (500 mL) and the combined methanolic extract was concentrated in vacuo to ca. ¼ of its original volume. The concentrate was diluted with water (200 ml) and applied to an open column packed with Supelco Discovery DSC-18Lt resin (ca. 150 g) in water. After the aqueous extract had been applied to the column 100 ml fractions employing a water-methanol gradient were collected. Fractions which were similar in composition were combined. All fractions and combined fractions were concentrated in vacuo to dryness and weight of residues determined. Fractions were analysed by HPLC-MS-PAD as outlined previously [17,18] (see supplementary data 2).

2.4 Purification of segetalin A, vaccarin, vaccaroside A and B, segetoside H, and segetoside I.

Segetalin A was obtained in pure form by dissolving segetalin A enriched fractions obtained from reverse phase chromatography in wet butanol-EtOAc (1:1) and washing with small amounts of 2% sodium bicarbonate solution. Evaporation of the butanol layer followed by chromatography on Amberchrom CG300M resin using a water-methanol gradient (20 –100%) afforded pure segetalin A as a white powder. The sample was homogeneous by TLC and HPLC and had spectral data consistent with its structure [19].

Vaccarin was obtained by rechromatographing vaccarin-rich fractions obtained from above on Amberchrom CG300M using a 0.001% NH_4OH-methanol gradient (0% MeOH to 50%) to afford vaccarin as a yellow powder [20].

Vaccaroside A was obtained by partitioning a vaccaroside A-enriched fraction obtained from a preparative fractionation on reverse-phase resin, between butanol and 0.1 M citric acid. The butanol portion was evaporated and the residue chromatographed on an Amberchrom CG300M resin using a water-methanol gradient (20 – 80%). The sample was determined to be free of bisdesmosides but did contain ca. 10% of other monodesmosides (including vaccaroside B MW 1278 and monodesmoside having MW 1294).

Vaccaroside B was obtained as for vaccaroside A and contained ca. 10% of other monodesmosides including vaccaroside A plus other monodesmosides. Vaccarosides A and B afforded spectral data consistent with their structures [21].

Segetoside H and I were obtained by re-chromatographing segetoside H- or I-enriched material which was obtained from earlier preparative fractionations, on

Amberchrom CG300M resin using a water-methanol gradient (40 – 100%) containing 0.01% acetic acid. Segetoside H and I were obtained as an amorphous white powders. The products were checked by HPLC-MS-PAD as described previously [17,18]. Segetoside H was greater than 90% pure, containing small amounts (<3% each) of other bisdesmosides including saponins having MW 1406, 1506, 1538, 1640. Segetoside I was obtained >85% purity having small amounts (<3% each) of saponins having MW 1598, 1436, 1506, and ca. 5% of saponin 1596. Spectral data obtained for segetoside H and I were consistent with their structures [22].

2.5 Measurement of growth inhibitory activity

Cell lines and cultures. Four human tumor cell lines were used for screening of *S. vaccaria* extracts for growth inhibitory activity: WiDr (ATCC # CCL-218) human colon cancer, MDA-MB-231 (ATCC # HTB-26) human breast cancer, NCI-417 (CRL-5809) human lung cancer and PC-3 (ATCC # CRL-1435) human prostate cancer. The human fibroblast cell line BJ (ATTC # CRL-2522) was used as non-tumorigenic control. Cell lines were initiated from stocks which were cryopreserved in liquid nitrogen. Stock cultures were maintained in RPMI Medium 1640 with L-glutamine supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Cells were grown in 20 mL media in T-75 flasks (Falcon) at 37^{o}C in humidified 5% CO_2. Typically, cells required feeding every 3-4 days and sub-culturing (passaging) once per week. Cells were always fed the day prior to harvest for the MTT experimental procedure. In preliminary experiments the number of cells required for seeding into each microwell to obtain the desired absorbance of about 0.300-0.400 was determined by plating cells at different densities. For cell lines WiDr, NCI-417 and BJ, 2.5×10^3 cells per well was chosen. For MDA-MB-231 and PC-3, 5×10^3 cells per well were used.

Preparation of assay plates for determination of IC_{50} values. Seed extracts were analyzed in triplicate and each assay was performed a minimum of three times. The growth inhibitory activities were evaluated at concentrations of 50, 25, 12.5, 6.3, 3.0 and 1.6 μg/mL saponin equivalent. Ninety microlitres (90μL) of the assay medium was added into wells that contained the highest concentration of plant extract. Culture medium (100 μL) was used as blank control. Negative control contained cells only. Positive controls employed cisplatin at doses similar to above. In general cisplatin consistently afforded sigmoid type curves with all the lines tested

however there was some variability in response with solutions in DMSO depending on age of solution. In general solutions were prepared within a few hours of use.

The highest concentration (50µg/mL) was prepared by adding 10 µL of the test extract solution into the first row of wells. Using a multichannel pipettor, 50µL two-fold serial dilutions for the extracts were performed. Plates were incubated for 48h at 37°C in a humidified 5% CO_2 atmosphere. Dye solution (15 µL) was added to each well and then incubated for additional 4h. Solubilization/Stop Solution (100 µL) was added to each well. Plates were allowed to stand overnight in a sealed container with a humidified atmosphere at room temperature to completely solubilize formazan crystals prior to data recording. The absorbance at 570nm was recorded using a 96-well microplate reader (ThermoLab System Multiskan Spectrum Microplate Spectro-photometer). The growth ratio of cancer cells under different concentrations of each plant extract was calculated using the formula Y (%) = $(1-T / C)\times 100$ where T = mean absorbance of treated cells and C = mean absorbance of negative control. Percentage mortality was plotted against the logarithmic values of the concentrations for calculation of IC_{50} values. IC_{50} is defined as the concentration of an extract or drug that is required for 50% inhibition of cell growth. These values were calculated from dose response curves generated by computer program MS Excel. Average values and standard deviations were determined using MS Excel statistics package.

2.6 Fluorescent Microscopy

Cells were grown on 22 mm^2 cover glasses in 6 well tissue culture plates. Following treatment with saponins, the cover glasses were rinsed once with PBS, and the cells were fixed for a minimum of 20 min in methanol kept at -20ºC. After rinsing with PBS, the cells were stained in PBS with 2 µg/mL Hoechst 33342 for 15 min. The cover glasses were washed five times with PBS, mounted on glass slides over PBS containing 50% glycerol and sealed with clear nail polish. The slides were observed using a Leica DMR microscope equipped with the Leica A4 filter. Images were captured using a DEI-750 CE Digital Output (Optronics).

2.7 Caspase 9 measurement

Cells were treated with 14 uM Segetoside H for various times and then processed according to Cell Technologies' APO LOGIX™ carboxyfluorescein caspase detection kit protocol using flow cytometry to detect activity. The assay was based on z-LEHD-FMK which is a potent inhibitor of caspase-9 (as well as –4 and –5). Flow cytometry was performed in Saskatoon at the Saskatchewan Cancer Agency's Cancer Research Unit using a Coulter Epics XL (Beckman). The kit was purchased from Cell Technology Inc, Mountain View, CA, USA.

2.8 *Apoptosis determination via Annexin V/propidium iodide staining*

Cells were treated with 7 uM segetoside I for various times and then processed according to Molecular Probes Vybrant™ apoptosis assay kit #2 protocol. The kit was purchased from Molecular Probes (Invitrogen), Eugene, OR, USA. Flow cytometry was performed in Saskatoon at the Saskatchewan Cancer Agency's Cancer Research Unit using a Coulter Epics XL (Beckman).

3. Results

3.1 Evaluation of the growth inhibitory activity of seed extracts from four varieties of Saponaria vaccaria L.

In order to ascertain if there was any differences in activity among *S. vaccaria* varieties, methanolic extracts of seed from wild type Scott (SWT), Pink Beauty (PB), White Beauty (WB) and Mongolia (MG) were prepared and evaluated on the basis of activity per amount of seed (deemed to contain 2% saponin by wt based on earlier studies[2]). The use of estimated saponin content of seed rather than the amount of extract obtained was due to the observation that most of the seed solubles were simple polysaccharides/sugars (raffinose, sucrose, glucose) which were somewhat variable in their extraction efficiencies under the protocol used. As these compounds are not growth inhibitory, their variable presence in the extracts could have masked varietal effects which were of interest. It was thus considered more informative to represent the IC_{50}'s as a function of the average saponin content of a *Saponaria* seed (ca. 2% w/w) which was *ex post facto* determined to be the most active components. In this way differences in activity of total seed extract could be

[2] saponin content of various batches of seed typically varied from 1.5 to 3% w/w; 2% represents an approximate average. The average amount of total extract obtained from seed varied from 6 –9 % by wt, depending on seed and conditions used.

ascribed to the variety rather than to the amount of extractables and would be more comparable to subsequent results obtained with purified saponins or saponin containing mixtures. Growth inhibitory activity was measured with the MTT assay initially using human cancer cell lines WiDr (colon), MDA-MB-231 (breast) and NCI-417 (lung). The activity of commercially available seed powder "Wang Bu Liu Xing" (WBX), *Quillaja* saponin (QS, estimated to be 100% saponin) and *Quinoa* saponin were also evaluated for comparative purposes. Cisplatin was used as positive control as it is an anticancer drug which is widely used clinically or experimentally against a variety of solid tumors. The results are summarized in Table 1 which lists the measured IC_{50}'s (as ug/mL saponin equivalent) and in Fig. 2 which shows dose response curves.

Overall, there was a dose-dependent response to *Saponaria* extract as indicated by growth inhibition with 100% effect at the highest concentration tested (50 ug/mL saponin equivalent). At lower doses, the highest sensitivity was exhibited by WiDr cells (IC_{50}'s 3.8 – 9.4 ug/mL, Table 1). The highest potency was exhibited by SWT extract (IC_{50}'s 3.8 – 12.6 ug/mL) followed by extracts of the other varieties (IC_{50}'s 7.7 – 18.7 ug/mL, Table 1) which were quite similar to each other against the particular line tested. *Quinoa* saponins did not exhibit activity, while *Quillaja* saponin extract (QS) although active was less potent (IC_{50}'s 17.9 – 28.9 ug/mL) than the *Saponaria* extracts. The commercial seed powder extract was similar in activity to the other *Saponaria* extracts with the exception of the more potent SWT.

3.2 Preparative reverse phase fractionation of seed extract

Although SWT variety seed was the most potent, seed from the PB variety was most readily available and was used for the preparative fractionation. This was deemed reasonable as its extract was also quite active and contained the same saponins and many of the other constituents as SWT, albeit in different amounts [18] (see Supplementary data 1). The 70% methanolic extract prepared from 100 g seed was fractionated on an open column containing a C_{18} reverse phase packing and a gradient of water-methanol (0 – 100%) was run. Fractions were analysed by HPLC-MS-PAD. This fractionation resulted in some discrimination between classes of compounds, although the various fractions obtained were still composed of complex mixtures of compounds. The early aqueous eluent contained mainly sugars and polar molecules. As methanol proportion increased flavonoids eluted

followed by monodesmosides, cyclopeptides, and bisdesmosides (see supplementary data 2).

Known cyclopeptides such as segetalins A, B, D, F, and G as well as some bisdesmosidic triterpene saponins (e.g. vaccaroside E, MW=1422; vaccaroside G, MW=1406; segetoside H, MW=1448; segetoside I, MW=1464) were readily identified in several fractions by HPLC-MS analysis using selected ion extraction of quasi-molecular ions from the total ion chromatogram (TIC) and confirmed by their extracted mass spectra. Monodesmosidic saponins mostly had MW <1200 with the exception of vaccaroside B (MW1278) and were readily identifiable by the presence of a dominant ion due to the sapogenin fragment (gypsogenic acid or hydroxygypsogenic acid, ESI^-, m/z 485 and 501 [17,18]. Many other bisdesmosidic saponins had previously been reported but only partially characterized and shown to be quillaic acid bisdesmosides having the same 3-O-disaccharide or trisaccharide sugars [18].

3.3 Isolation and biological evaluation of some purified components

Fractions which were enriched in various components were further processed by solvent-solvent partitioning using pH control, and rechromatography on a different reverse phase resin using appropriate gradients. In this manner flavonoid vaccarin, cyclopeptide segetalin A, monodesmosides vaccarosides A and B, as well as bisdesmosides segetoside H and I were obtained in relatively pure form (ca >85% by HPLC) (Fig. 1).

The above compounds were evaluated for growth inhibitory activity using the MTT assay with human cancer cell lines WiDr, MDA-MB-231, NCI-417 and PC-3 (prostate) as well as with the human fibroblast cell line BJ (CRL-2522). In addition *Kochia* saponin (from seed of *Kochia scoparia* (L.) Roth), was available [17] and included for comparison purposes. The IC_{50} results are summarized in Table 4. The monodesmosides vaccaroside A and B, cyclopeptide segetalin A, and *Kochia* saponins did not exhibit activity. Segetoside H and I, both bisdesmosidic *Saponaria* saponins exhibited good activity as illustrated by the dose-response curves in Fig. 3. The most sensitive cell line to both compounds was the MDA-MB-231 (IC_{50}'s of 1.3 ug/ml = 0.90 uM, segetoside H and 1.6 ug/ml = 1.09 uM, segetoside I) and the least sensitive was the NCI-417 (IC_{50}'s of 18.6 ug/mL = 12.8 uM, segetoside H and 19.8

ug/mL = 13.5 uM , segetoside I) closely followed by the WiDr cell line (15.9 ug/ml = 11.0 uM, segetoside H and 16.7 ug/ml = 11.4 uM, segetoside I).

3.4 Microscopy of cells treated with Segetoside H

To further investigate the effect of *Saponaria* saponins on different cells, microscopic examination was performed. Segetoside H was the most easily obtained pure saponin, so initial microscopy utilized this compound. Treatment of MDA-MB-231 breast cancer cells and PC-3 prostate cancer cells with 7 uM segetoside H for 20 h followed by staining with the nucleus specific fluorescent Hoescht 33342 dye (Fig. 4 and 5) followed by microscopic analysis indicated significant morphological changes to both cells and nuclei in the treated samples. These cancer cells, which were found to be the most sensitive to the saponins by the MTT assay, exhibited many condensed and sickle shaped nuclei, typical of cells undergoing apotosis. In phase contrast mode there were clearly visible differences between treated and untreated cells, with treated cells showing a 'rounded' morphology.

In contrast to the tumorigenic cell lines, treatment of the non-tumorigenic fibroblast cell line, BJ (CRL-2522), with segetoside H did not lead to noticeable differences between the treated and untreated samples (Fig. 6). Thus, although growth inhibition of the BJ line by segetoside H at low micromolar concentrations was indicated by the MTT assay, the treated cells did not exhibit any morphological differences from untreated cells at a comparatively high dose.

3.5 Caspase 9 Activity

Additionally, MDA-MB-231, PC-3 and BJ cells treated with 14 μM Segetoside H for various times were analyzed for apoptotic activity using the APO LOGIX™ Carboxyfluorescein Caspase 9 Detection Kit using flow cytometry. Both MDA-MB-231 and PC-3 cells treated with segetoside H showed increasing numbers of cells with caspase 9 activity relative to untreated cells, with the effect showing up in as little as 10 hrs. The effect was time dependent with maximum caspase 9 activity after 48 hr exposure for the MDA-MB-231 cell line. In contrast BJ cells were largely unaffected even at 48 h. The results are shown in Fig. 7.

3.6 Apotosis determination with Annexin V/Propidium iodide staining

Segetoside I was also evaluated for induction of apoptosis in PC-3 and BJ cells by using Annexin V/propidium iodide staining and flow cytometry to detect cell death and cells undergoing apoptosis. The time course using 7 uM segetoside I with PC-3 cells is shown in Fig. 8. BJ cells were largely unaffected by 7 uM segetoside I over the 36 h time course (data not shown), whereas the number of apoptotic cells increased in the PC-3 treated cells in a time dependent manner.

4. Discussion

The evaluation of *S. vaccaria* for antiproliferative activity initially involved using the MTT assay, with total extracts derived from seed of several varieties, followed by fractionation to obtain fractions of mixed components followed by further fractionation/purification to obtain several pure components. The results were different depending on the material used in the assays. When the total seed extract was used, the most sensitive cell line was the WiDr; but when bisdesmosidic saponin containing fractions (see Supplementary data 3) or purified bisdesmosidic saponins segetoside H and I were used, the MDA-MB-231 cell line was more sensitive to these compounds by an order of magnitude than the WiDr cells. These results indicated that there were likely some interactions among the various seed components in the total extracts which affected the response of the cells to these saponins. However, it is not unusual for a complex extract to exhibit a somewhat different bioactivity profile as compared to the pure components. Regardless of any interactive effects however, the main growth inhibitory activity appeared to be due to the bisdesmosidic saponins as evidenced by lack of activity (IC_{50}'s > 50 ug/ml) of the purified monodesmosides vaccarosides A and B, cyclopeptide segetalin A, or phenolic vaccarin. By comparison the purified bisdesmosides, Segetoside H and I, exhibited IC_{50}'s at the low micromolar level similar to the clinically utilized drug cisplatin which was used as the positive control.

Interestingly, the results reported here are contradictory to Ma et al's [13] report of growth inhibitory activity by monodesmoside vaccaroside B, but confirmatory of their observed potent activity of several bisdesmosidic saponins. It is possible that the cell lines they tested possessed different sensitivity than the ones used here, but it is felt more likely that the vaccaroside B used in their assay may have been contaminated with some bisdesmosidic saponins. Separation of saponins from each other is quite difficult as they tend to form mixed aggregates and

are structurally quite closely related [23]. The monodesmosides, vaccarosides A and B tested here were not contaminated with bisdesmosidic saponins as specifically determined from HPLC-MS analysis of the samples tested, although they did contain small amounts of other monodesmosides.

In the varietal comparison, *Saponaria* extracts with the exception of the SWT extract were quite similar in potency and selectivity in the dose-related growth inhibition of cells. The SWT extract which was previously observed to contain a larger proportion of higher molecular weight saponins (generally having a 3-*O*-trisaccharide structure, as compared to the 3-O-disaccharide homologs) [18], was found to be significantly more potent on a seed weight basis. This greater potency could have been due to profile differences between varieties, but it could simply have been due to a higher titer of active saponins, as the comparison was based on the assumption of 2% seed saponin content (the extracts were prepared from the same amount of seed of each variety and serially diluted). Regardless of the reasons, the SWT variety clearly produced seed which was more “active” on an equivalent weight basis than the other varieties. Although not directly relevant it was interesting to note that the seed of this variety was larger than the others and the plant tended to be the most prolific seed producer as well. It thus represents an attractive plant for further breeding or development work.

Bisdesmosidic *Saponaria* saponins are mainly quillaic acid derivatives with a minority being derivatives of the closely related triterpene gypsogenin. The importance of these sapogenins to the activity was indicated by the fact that the structurally similar Q*uillaja* saponins were also observed to possess growth inhibitory activity, albeit at a lower level (i.e. less potent). Further confirmation for the importance of quillaic acid’s general role in the growth inhibitory activity is also available in the literature. For example, Bai *et al* [24] reported a quillaic acid bisdesmoside from *Gypsophila oldhamiana* to be growth inhibitory to HT-29 (colon), SGC7901(gastric), and PLC/PRF/5(hepatoma) cell lines at low micromolar concentrations. In addition, in our hands neither Q*uinoa* saponins, which are a mixture of mono- and bisdesmosides having phytolaccagenic acid, hederagenin, and oleanolic acid sapogenins [25], nor *Kochia* saponins, which are a mixture of mono- and bisdesmosides derived from oleanolic acid [17] were active at the levels tested. The presence of quillaic acid or gypsogenin as the sapogenin thus appears to be a significant contributing factor to antiproliferative activity.

The non-tumorigenic BJ (CRL-2522) human fibroblast line was evaluated against various saponin-containing fractions (see supplementary data 3) as well as segetosides H and I in order to ascertain if some selectivity between tumorigenic and non-tumorigenic cells would be observed. In the growth inhibition studies this was not the case, as the various extracts, bisdesmosidic saponins and saponin-containing fractions tested affected growth inhibition in this line at similar doses to the tumor lines. However, in investigating morphological effects, caspase 9 induction, and Annexin V/propidium iodide binding, distinct differences were noted in that the non-tumorigenic fibroblast line did not exhibit any morphological changes to cells or nuclei, nor little caspase induction at the comparatively high dose of 14 uM segetoside H. These cells also did not show cell death or apoptosis induction on treatment with 7 uM segetoside I over a 36 h time course, as determined *via* Annexin V/propidium iodide staining (data not shown). In contrast to the BJ cells, PC-3 cells exhibited increasing time-dependent Annexin V and propidium iodide binding, indicative of apoptotic and/or dying cells when treated with segetoside I at 7 uM. It thus appeared that while bisdesmosidic saponins inhibited the growth of the BJ cells they did not induce apoptosis at those dosage levels. In contrast, the tumorigenic lines MDA-MB-231 and PC-3, on treatment with 7 to 14 uM segetoside H or I, underwent significant morphological changes, production of caspase 9, and Annexin V binding consistent with cells undergoing apoptotic cell death. It thus appeared that the bisdesmosides could selectively induce apoptosis in tumorigenic cells.

In conclusion, bisdesmosidic *Saponaria* saponins, in contrast to monodesmosidic *Saponaria* saponins, cyclopeptides, or C-glycosylflavonoids, were found to possess potent growth inhibitory and selective apoptosis inducing activities and are likely the main contributors to the antiproliferative activity of seed extracts.

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LIST OF TABLES

Table 1.

Cow cockle variety (70% MeOH extract)	IC_{50} based on estimated saponin content (ug/mL)*		
	WiDr	**MDA-MB-231**	**NCI-417**
Scott WT	3.8 ± 0.6	11.4 ± 1.3	12.6 ± 4.3
Pink Beauty	9.1 ± 0.3	18.4 ± 0.2	17.8 ± 1.9
Mongolia	7.7 ± 0.25	16.8 ± 0.6	15.2 ± 3.2
White Beauty	9.4 ± 1.1	19.6 ± 0.1	18.4 ± 0.6
Wang Bu Liu Xing (Botanicum brand)	9.5 ± 1.0	19.4 ± 0.1	18.7 ± 2.1
Quillaja saponin	17.9 ± 0.7	22.9 ± 1.1	28.9 ± 9.9
Quinoa saponin	> 50	> 50	> 50
Cisplatin (positive control)	2.5± 2.6 (8.1± 8.4) **	2.6± 2.3 (8.6± 7.6)	1.3± 1.3 (4.4± 4.5)

*Growth inhibition was determined using total seed extract but expressed as saponin equivalent ± S.D. performed in triplicate in at least two independent experiments.

** Number in brackets refers to IC_{50} in uM

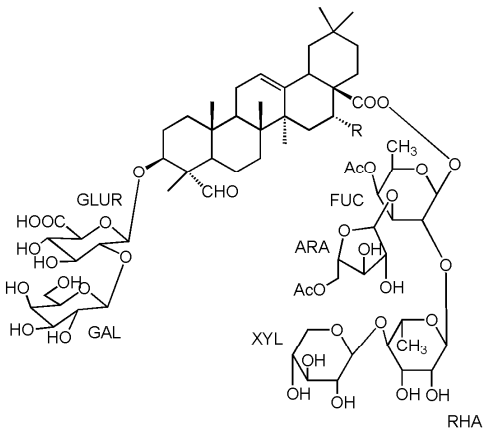
Table 2

Cell line / Sample	WiDr	MDA-MB-231	NCI-417	PC-3	CRL-2522
Vaccaroside A (MW 1134)	ND	>50	ND	>50	>50
Vaccaroside B (MW 1278)	> 50	> 50	> 50	N/D	> 50
Segetoside H (MW 1448)	15.9 ± 0.5 (11.0 ± 0.3)	1.3 ± 0.1 (0.9 ± 0.1)	18.6 ± 2.3 (12.8 ± 1.6)	11.8 ± 3.8 (8.1 ± 2.6)	14.1 ± 1.4 (9.7 ± 1.0)
Segetoside I (MW 1464)	16.7 ± 1.0 (11.4 ± 0.7)	1.6 ± 0.6 (1.1 ± 0.4)	19.8 ± 0.2 (13.5 ± 0.1)	4.0 ± 1.4 (2.7 ± 1.0)	4.0 ± 1.3 (2.7 ± 0.9)
Vaccarin (MW 726)	> 50	> 50	> 50	>50	> 50
Segetalin A (MW 609)	> 50	> 50	> 44	N/D	> 50
Kochia saponin	> 50	> 50	> 50	N/D	> 50
Cisplatin (positive control)	2.1± 0.8 (6.9± 2.8)	6.1± 4.0 (20.0± 13.1)	2.6± 1.7 (8.6± 5.5)	2.2± 1.2 (7.2± 4.1)	2.9± 0.9 (9.5± 3.0)

N/D, not done

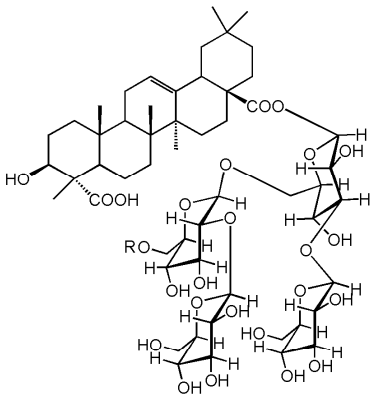
LIST OF FIGURES

Fig. 1



R = H, Segetoside H, MW 1448

R = OH, Segetoside I, MW 1464



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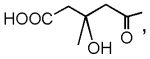
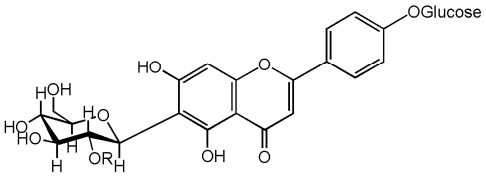
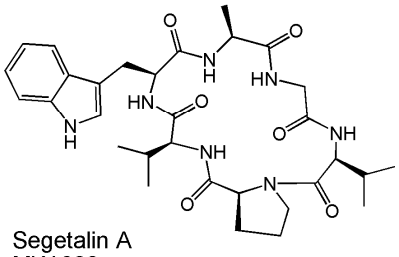
R = 

, Vaccaroside B, MW 1278



R = Arabinose, Vaccarin, MW 726

cyclo (gly-val-pro-val-trp-ala)



Segetalin A
MW 609

Fig. 2

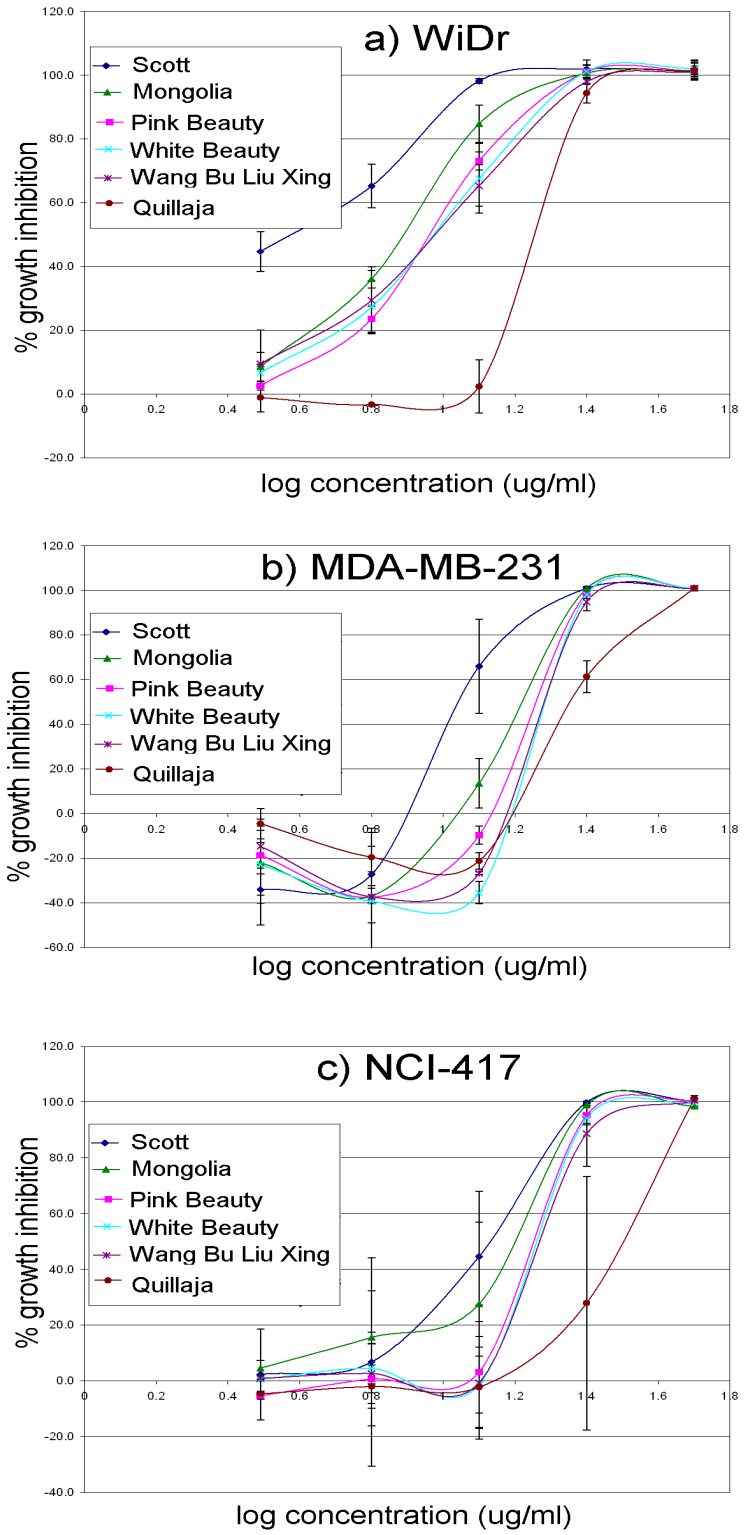

a) WiDr
Scott
Mongolia
Pink Beauty
White Beauty
Wang Bu Liu Xing
Quillaja
% growth inhibition
log concentration (ug/ml)
b) MDA-MB-231
Scott
Mongolia
Pink Beauty
White Beauty
Wang Bu Liu Xing
Quillaja
% growth inhibition
log concentration (ug/ml)
c) NCI-417
Scott
Mongolia
Pink Beauty
White Beauty
Wang Bu Liu Xing
Quillaja
% growth inhibition
log concentration (ug/ml)

Fig. 3

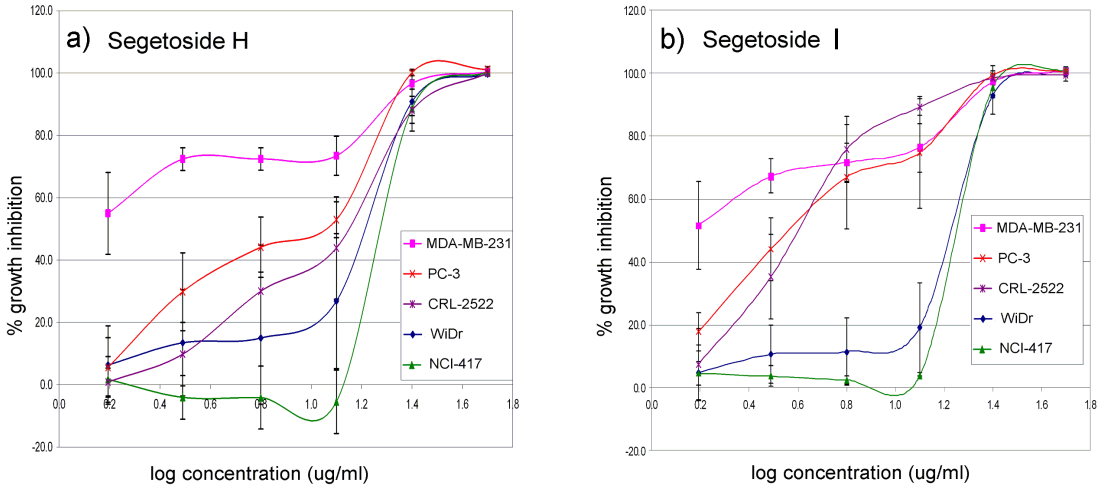

a) Segetoside H
% growth inhibition
log concentration (ug/ml)
MDA-MB-231
PC-3
CRL-2522
WiDr
NCI-417
b) Segetoside I
% growth inhibition
log concentration (ug/ml)
MDA-MB-231
PC-3
CRL-2522
WiDr
NCI-417

Fig. 4

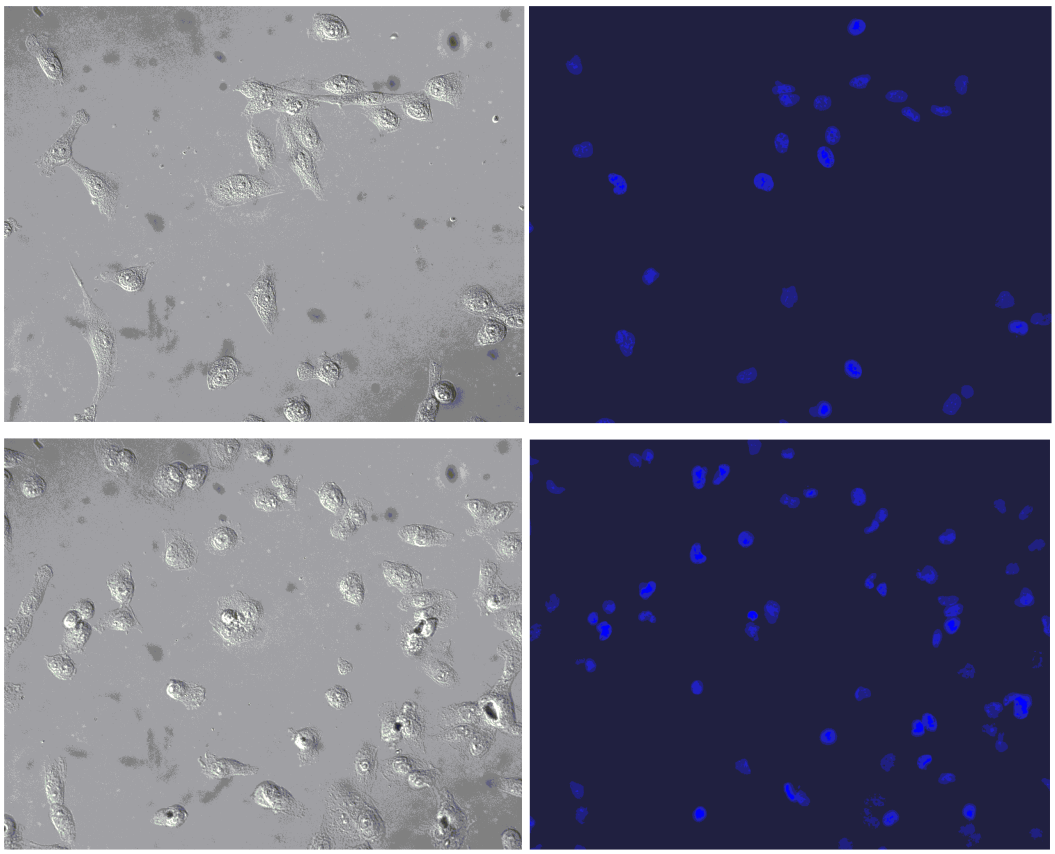


Fig. 5

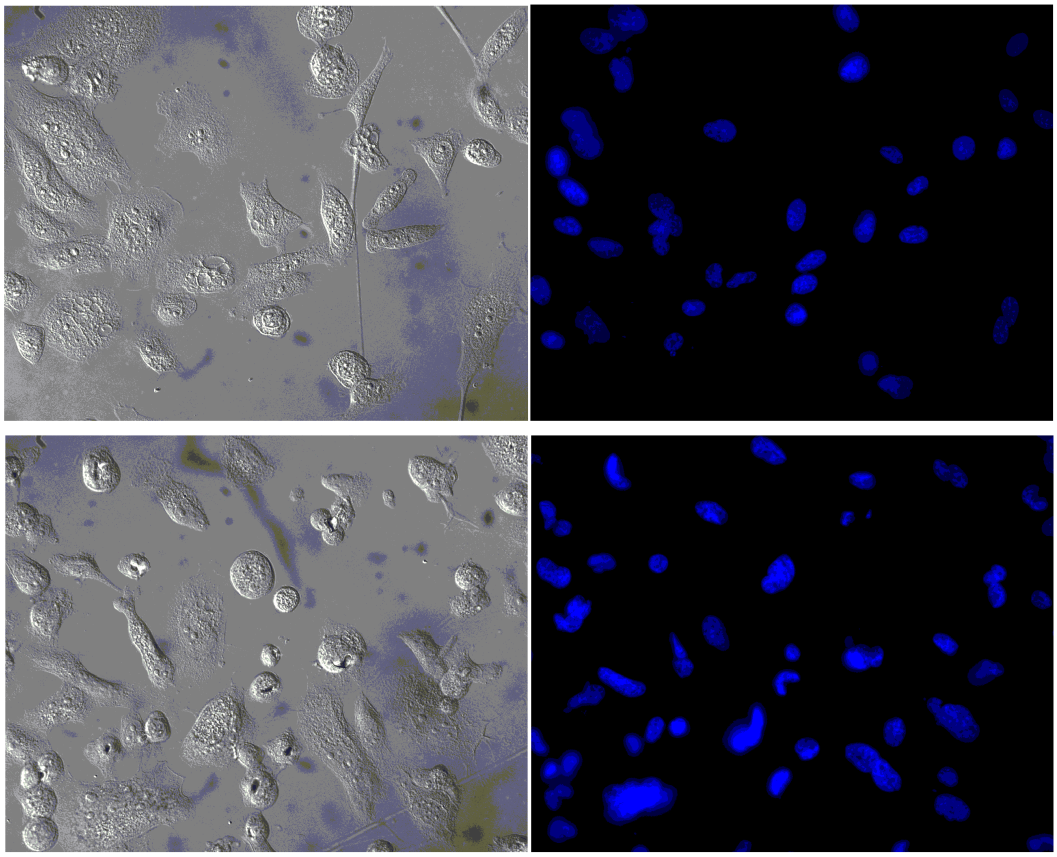


Fig. 6

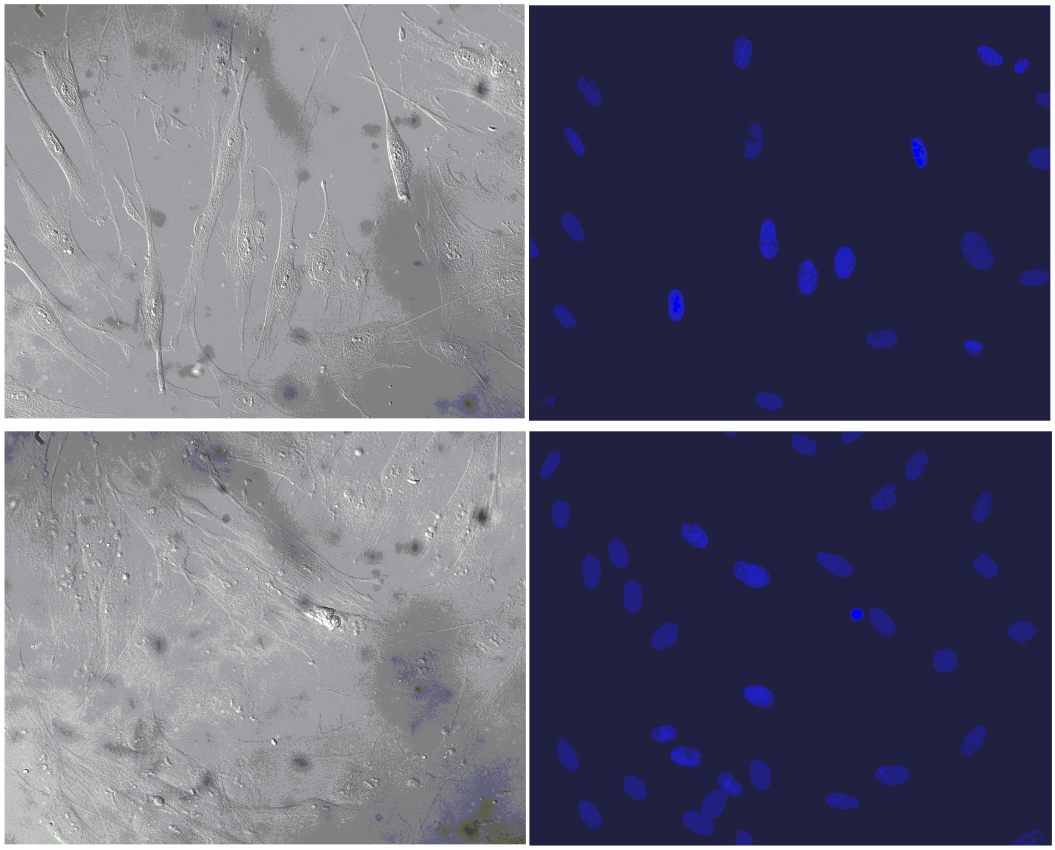
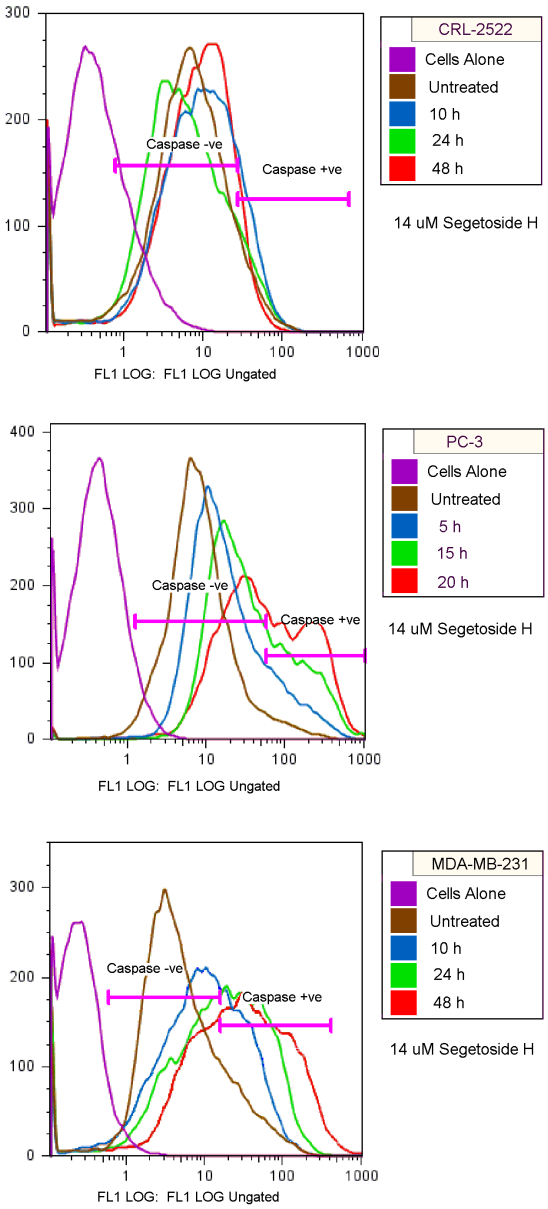


Fig. 7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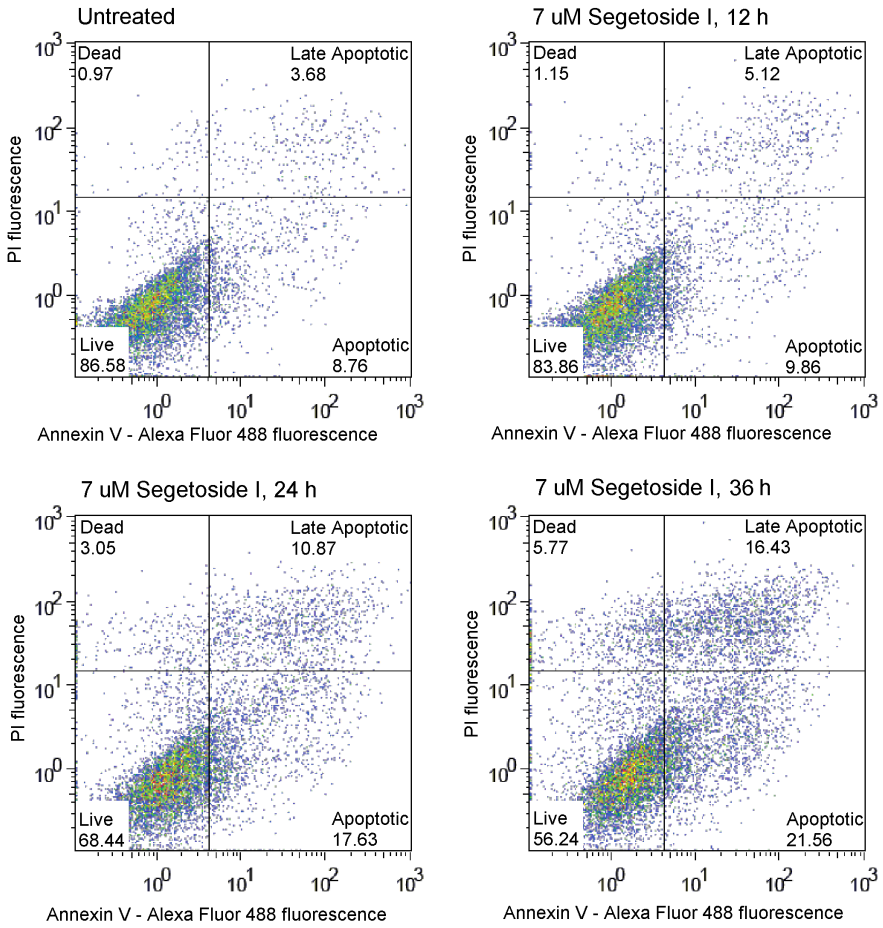
200
100
0
Caspase -ve
Caspase +ve
1
10
100
1000
FL1 LOG: FL1 LOG Ungated
CRL-2522
Cells Alone
Untreated
10 h
24 h
48 h
14 uM Segetoside H
400
300
200
100
0
Caspase -ve
Caspase +ve
1
10
100
1000
FL1 LOG: FL1 LOG Ungated
PC-3
Cells Alone
Untreated
5 h
15 h
20 h
14 uM Segetoside H
300
200
100
0
Caspase -ve
Caspase +ve
1
10
100
1000
FL1 LOG: FL1 LOG Ungated
MDA-MB-231
Cells Alone
Untreated
10 h
24 h
48 h
14 uM Segetoside H

Fig. 8


Untreated
Dead
0.97
Late Apoptotic
3.68
Live
86.58
Apoptotic
8.76
PI fluorescence
Annexin V - Alexa Fluor 488 fluorescence
7 uM Segetoside I, 12 h
Dead
1.15
Late Apoptotic
5.12
Live
83.86
Apoptotic
9.86
PI fluorescence
Annexin V - Alexa Fluor 488 fluorescence
7 uM Segetoside I, 24 h
Dead
3.05
Late Apoptotic
10.87
Live
68.44
Apoptotic
17.63
PI fluorescence
Annexin V - Alexa Fluor 488 fluorescence
7 uM Segetoside I, 36 h
Dead
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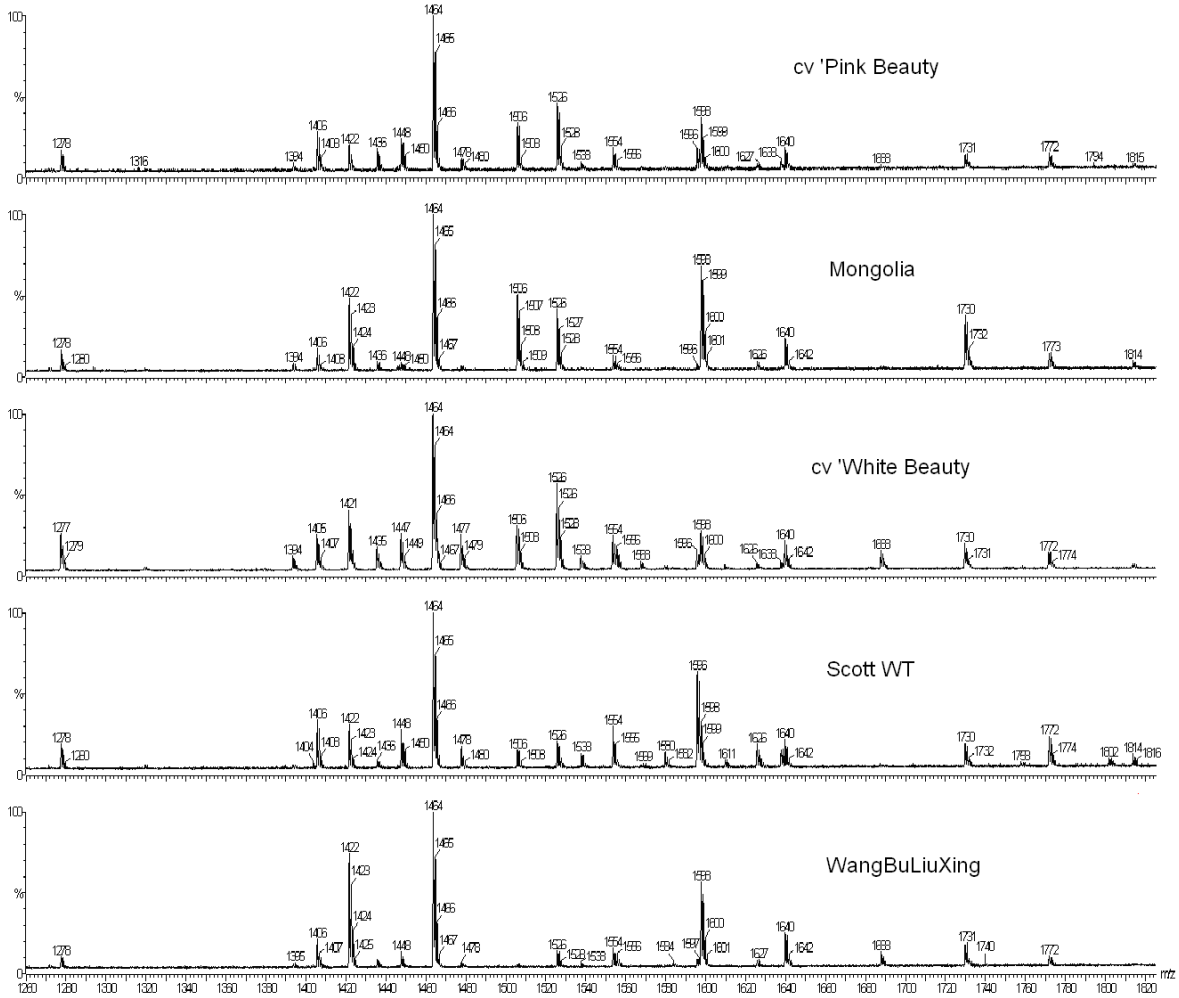
Late Apoptotic
16.43
Live
56.24
Apoptotic
21.56
PI fluorescence
Annexin V - Alexa Fluor 488 fluorescence

Supplementary data1:



Bisdesmosidic (and vaccaroside B) saponin profles via extracted MS (m/z 1250 – 1820) obtained from HPLC-MS analysis of of seed from various *Saponaria vaccaria* accessions.

Supplementary data 2:

Fraction No.	% MeOH	Yield and observations	Composition
1-7	0 -20	5 g	mainly sugars, polar compounds
8/9	25	ca. 100 mg	MW 520 sucrose ester (dihydrocinnamate)
10/11	30-35	226 mg, yellow	vaccarin (MW 726) + tryptophan betaine (MW 246)*
12/13	40	60 mg, yellow	flavonoids (MW 726, MW 564) + monodesmosidic saponins
14	45	87 mg, yellow	flavonoids (MW 564, 904) + mono- and bisdesmosides
15/16	45-50	215 mg	segetalin B (MW 484) + mono- and bisdesmosides
17/18	50-55	F 17-20 540 mg	segetalins A (MW 609), F (MW 954), G (MW 519) + mono- and bisdesmosides
19/20	55-60		
21/22	60-65	328 mg	segetalin D (MW 719) and bisdesmosidic saponins
23/24	70	380 mg	mainly segetoside I (MW1464) + other bisdesmosides
25/26	80	1.2 g	mainly bisdesmosidic saponins and vaccaroside B (MW 1278)
27/28*	80	280 mg	** cryst. mainly bisdesmosidic saponins (MW 1640, MW 1772); ML (mother liq.), mainly segetoside H (MW 1448)

* Tryptophan betaine had not been previouslty reported as a seed constituent. It was identified by UV, NMR and LC-MS.

** On standing some saponins in fraction 27/28 precipitated from solution – referred to as 27/28 cryst. Saponins remaining in solution were referred to as 27/28 ML.

Summary of initial fractionation on Discovery DSC-18Lt resin using water-methanol gradient.

Supplementary data 3:

Cell line / Sample fraction	WiDr (colon)	MDA-MB-231 (breast)	NCI-417 (lung)	CRL-2522 (fibroblast)
Fraction 1 - 13	> 50	> 50	> 50	N/D
Fraction 14	46.2 ± 1.0	26.2 ± 16.4	> 50	N/D
Fraction 15-16	8.7 ± 1.4	4.6 ± 2.3	17.9 ± 1.1	5.3 ± 0.2
Fraction 17-20	11.3 ± 2.7	10.1 ± 4.2	22.6 ± 4.6	6.3 ± 0.1
Fraction 21-22	10.0 ± 2.0	10.7 ± 2.8	16.4 ± 2.8	3.6 ± 0.3
Fraction 23-24	11.8 ± 3.3	9.5 ± 3.1	19.5 ± 3.8	7.7 ± 0.5
Fraction 25-26	8.4 ± 2.2	6.7 ± 2.1	13.4 ± 2.2	5.0 ± 0.3
Crystal fraction 27-28	8.8 ± 2.3	12.3 ± 2.2	13.9 ± 1.9	4.7 ± 0.1
ML 27-28	12.8 ± 3.1	10.2 ± 3.0	17.3 ± 4.7	N/D
Quillaja saponin	24.4 ± 7.4	28.3 ± 9.3	27.3 ± 6.9	N/D

N/D, not done

Growth inhibitory activity (IC_{50}'s) of fractions obtained from initial separation.