Comparative evaluation of *in-vitro* cytotoxicity, antiviral and antioxidant activities of different soyasapogenols from soybean saponin

Hala A. Amin[a], Hanem M. Awad[b] and Atef G. Hanna[c]

Departments of [a]Chemistry of Natural and Microbial Products, [b]Tanning Materials and Leather Technology and [c]Chemistry of Natural Compounds, National Research Center, Cairo, Egypt

Correspondence to Hala A. Amin, Department of Chemistry of Natural and Microbial Products, National Research Center, Dokki, 12622 Cairo, Egypt
Tel: +20 2 33464472; fax: +20 2 37622603; e-mail: halaamin2007@yahoo.com

Revised 15 February 2012
Accepted 7 June 2012

Egyptian Pharmaceutical Journal
2012,11:73–79

Objectives
The aim of this study was to evaluate comparative and structure–activity relationships of *in-vitro* cytotoxicity, antiviral and antioxidant activities of soyasapogenols A, B, D and F (SSA, SSB, SSD and SSF) together against the total soyasaponin extract (TSSE) itself.

Methods
The cytotoxicity of soyasapogenols and TSSE against human colon carcinoma cell line (HCT-116), liver carcinoma cell line (Hep-G2), human breast carcinoma cell line (MCF-7) and normal human melanocytes (HFB-4) cell lines was assessed using sulforhodamine B assay. Their antiviral activities were investigated against Rift Valley fever virus (RVFV), hepatitis C virus model (vesicular stomatitis virus, VSV), and hepatitis A virus (HAV). The antioxidant activity of soyasapogenols and TSSE was assessed using a stable DPPH free radical.

Results and conclusion
The results obtained showed that both TSSE and soyasapogenols have a potent cytotoxic effect on Hep-G2, HCT-116, MCF-7 and HBF-4 cell lines in a concentration-dependent manner. SSA and SSF showed the highest cytotoxic activities against tested cell lines. Analysis of the three-dimensional structure of the measured soyasapogenols indicated that if the β-hydroxyl group at C-21 or C-22 was aligned with the plane of the molecule, a marked increase in the cytotoxic activity of the soyasapogenol was produced. Their antiviral activities against RVFV, VSV and HAV showed significant inhibition activities compared with both TSSE and interferon. SSB showed the best activity against RVFV and HAV, whereas SSA was the best inhibitor against VSV. It was concluded that the hydroxylation at C-21 as well as the presence of a double bond in ring D might enhance anti-VSV activity, whereas they may not be essential for anti-RVFV and anti-HAV activities. On the other hand, the tested soyasapogenols and TSSE did not show good antioxidant activities.

Keywords:
antioxidant, antiviral activity, cytotoxicity, soyasapogenols, soyasaponins

Egypt Pharm J 11:73–79

1687-4315

Introduction

Soyasaponins are major phytochemical compounds present in legume seeds [1], soybeans and soy products [2]. The basic structure of soyasaponins is an oleanene-type triterpenoid aglycone to which one or more polysaccharide chains are attached, resulting in the amphiphilic nature of the molecules. They are divided into three groups, on the basis of the structure of the aglycone moiety, A, B and E saponins [3]. Soyasapogenols are the aglycone moieties of soyasaponins. They can be obtained by acid or alkali hydrolysis of soyasaponins or by enzymatic hydrolysis using microorganisms with soyasaponin-hydrolyzing activity [4–6].The current consensus is that soyasapogenols A, B, and E (SSA, SSB and SSE) are true aglycones, whereas C, D and F (SSC, SSD and SSF) are artifacts produced during the hydrolysis process [7]. Soyasaponins have been reported to have several health-beneficial activities including hepatoprotective [8], antiviral [9], anticarcinogenic [10], antioxidant [11] and anti-inflammatory activities [12]. Recent studies have shown that a total soyasaponin extract (TSSE) can inhibit the growth of hepatocarcinoma (Hep-G2) cells [4], colon adenocarcinoma cells (HCT-15) [13] and cervical tumor (Hela) cells [14] by inducing programmed cell death, either apoptosis, or microautophagy [15]. Soyasapogenols have been shown to be more effective than their glycosides in the suppression of 2-acetoxyacetylaminofluorene (2-AAAF)-induced genotoxicity in Chinese hamster ovary cells [16]. Both SSA and SSB have shown almost complete suppression of HT-29 colon cancer cell growth. Moreover, soyasaponins might be an important dietary chemopreventive agent against colon cancer after alternation by microflora [17]. Both SSA-containing and

1687-4315 © 2012 Division of Pharmaceutical and Drug Industries Research, National Research Centre
DOI: 10.7123/01.EPJ.0000421669.78647.e9

SSB-containing extracts have also been reported to be capable of inducing apoptosis. SSA extract-treated Hep-G2 cells were reported to induce 47 ± 3.5% of the cells to undergo apoptosis, whereas SSB extract induced 15 ± 4.2% of cells to undergo apoptosis after 72 h of treatment [4]. In addition, SSB (10 μmol/l) was growth inhibitory to MDA-MB-231 human breast cancer cells *in vitro* [18].

SSA, SSB, SSE and soyasaponin I, a major constituent of group B saponins, completely inhibited HIV-induced cytopathic effects 6 days after infection at a concentration greater than 0.25 mg/ml, but exerted no direct effect on HIV reverse transcriptase activity [19]. TSSE showed a significant inhibitory effect on the replication of HSV-1 and CoxB3 [20]. In a structure–activity relationship study, the activity of SSA was less than 1/20 of that of SSB and the hydroxylation at C-21 seemed to reduce anti-HSV-1 activity [21]. Soyasaponin II was found to inhibit the replication of the human cytomegalovirus and influenza virus. This action was not because of the inhibition of virus penetration and protein synthesis, but may because of a virucidal effect [22]. The effect of TSSE from soybean on acute alcohol-induced hepatotoxicity in mice has been investigated. Mice treated with TSSE showed a better profile of the antioxidant system with normal superoxide dismutase, glutathione S-transferase, and glutathione peroxidase activities, which were associated with the increase in hepatic glutathione levels relative to the acute alcohol-treated group [23]. However, TSSE and its five main constituent saponins had a much weaker *in-vitro* inhibitory effect on lipid peroxidation induced by NADPH in mouse liver microsomes than α-tocopherol [11].

All of these reports have led to our interest in a comparative evaluation of *in-vitro* cytotoxicity, antiviral and antioxidant activities of SSA, SSB, SSD and SSF isolated from a hydrolyzed soybean saponin extract against TSSE itself and to discuss their structure–activity relationships.

Materials and methods

Materials

Soybean saponin (50%) was purchased from Organic Technologies Co. Ltd (Coshocton, Ohio, USA). SSA, SSB, SSD and SSF were isolated from a hydrolyzed soybean saponin extract [6]. Sulforhodamine B (SRB), Roswell Park Memorial Institute (RPMI) 1640 medium, and 1,1-diphenyl-2-picryl hydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). Fetal bovine serum (FBS), 199 E-Hepes buffer medium and fetal calf serum (FCS) were purchased from Gibco (Paysley, UK). Recombinant human interferon α2a (rh-IFN α2a) was obtained from Galaxo Smithkline (Milan, Italy). Dimethyl sulfoxide (DMSO) and methanol were of HPLC grade, and all other reagents and chemicals were of analytical reagent grade. To determine the structure–activity relationships, the three-dimensional (3D) structure of the measured compounds was created using VEGA ZZ software (Drug Design Laboratory, University of Milan, Milan, Italy), and energy minimization was carried out by AMMP calculation provided by the same software.

Cell culture

Four human cell lines, HCT-116 (colon carcinoma cell line), Hep-G2 (liver carcinoma cell line), MCF-7 (human breast carcinoma cell line) and HFB-4 (normal human melanocytes) were purchased from the American Type Culture Collection (Rockville, Maryland, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 U/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Cytotoxic activity (sulforhodamine B assay)

Human cancer cell lines were grown in RPMI-1640 medium (37°C, 5% CO_2) to assess the growth inhibition by a colorimetric assay, which estimates the cell number indirectly by staining total cellular protein with SRB dye [24]. Logarithmically growing cells were seeded at a density of 10^4 cells/well into 96-well plates and allowed to adhere for 24 h at 37°C. Then, the supernatant was replaced by 100 μl culture medium supplemented with each tested compound in DMSO at specified concentrations and incubated at 37°C for 48 h. The final concentration of DMSO in the solution in each well was 0.5%. Treatment with DMSO only was always used as a control. At the end of the treatment, the supernatant from each well was discarded and cells were fixed by layering 100 μl ice-cold 15% trichloroacetic acid on top of the growth medium. They were then incubated at 4°C for 1 h. The plates were then washed five times with cold water, the excess water was drained off, and the plates were air dried. SRB stain [100 μl; 0.4 (w/v) in 1% acetic acid] was added to each well and left in contact with the cells for 1 h. Subsequently, the cells were washed with 1% acetic acid and rinsed four times. The plates were dried, and 1 ml of 10 mmol/l Tris base was added to each well to dissolve the dye. The plates were shaken gently for 20 min on a gyratory shaker, and the absorbance (OD) of each well was read on a spectrophotometer at 540 nm. Cell survival was measured as the percentage of absorbance compared with the control.

DPPH radical-scavenging assay

The antioxidant activity of soyasapogenols (SSA, SSB, SSD, and SSF), TSSE and standards (ascorbic acid and rutin) was assessed on the basis of the radical-scavenging effect of a stable DPPH free radical [25]. A volume of 10 μl of each tested compound or standard (from 0.0 to 100 μg/ml) was added to 90 μl of a 100 μmol/l methanolic solution of DPPH in a 96-well microtiter plate (Sigma-Aldrich Co.). After incubation in the dark at 37°C for 30 min, the decrease in the absorbance of each solution was measured at 520 nm using an ELISA micro plate reader (Model 550; Bio-Rad Laboratories Inc., Hercules, California, USA). The absorbance of the blank sample containing the same amount of DMSO and DPPH solution was also prepared and measured. All experiments

were carried out in triplicate. The scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid. Radical-scavenging activity was calculated using the following formula:

$$\% \text{ Reduction of absorbance} = [(AB - AA)/AB] \times 100,$$

where AB is the absorbance of the blank sample and AA is the absorbance of the tested compound ($t = 30$ min) [26].

Antiviral activity

Cells and viruses

Vero clone CCL-81 was obtained from the Cell Culture Department, VACSERA (Cairo, Egypt). Cells were grown in 199 E-Hepes buffer growth medium supplemented with 10% inactivated FCS, 5 mmol/l Hepes buffer, and antibiotics (100 U of penicillin/ml and 100 g of streptomycin/ml) at 37°C and incubated in a 5% CO_2 atmosphere. Vesicular stomatitis virus (hepatitis C virus model, VSV, Indiana strain), Rift Valley fever virus (RVFV, Menya/sheep/258) and hepatitis A virus (HAV, a local isolate) were kindly supplied by Applied Research Sector, VACSERA. The infectivity titer of the viruses was determined according to the reported method of Specter *et al.* [27]. The viruses were 10-fold serially diluted and each dilution was dispensed as 100 µl/well onto precultured Vero cells. Noninfected wells were considered as a negative control. Plates were incubated at 37°C. Seven days after infection, the 50% cell culture infective dose end point ($CCID_{50}$) was determined.

Cytotoxicity assay

The investigated compounds were dissolved in DMSO and diluted with sterile culture medium at specified concentrations. The cytotoxicity assay of each compound compared with sterile rh-IFN α2a was carried out according to previous reports [28,29], and a negative cell control was included. Plates were incubated at 37°C for 24 h. Cell culture-treated plates were examined microscopically using an inverted microscope for the detection of cellular changes or cytotoxicity. The medium was discarded and plates were washed using phosphate-buffer saline (pH 7). Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to determine the safe concentration range for each compound. Viability percentage was determined as follows:

$$\text{Viability}\,\% = (OD_{control} - OD_{treated})/OD_{control}) \times 100,$$

Antiviral activity

The antiviral activity of tested compounds and interferon against RVFV, VSV, and HAV was determined, where nontoxic concentrations of each compound and rh-IFN (10 IU/0.1 ml) as a positive control were used for the treatment of precultured Vero cells for 24 h. A negative cell control plate was included for viral control titration. Viruses were 10-fold serially diluted in 199 E-Hepes buffer (10^{-1}–10^{-8}). Antiviral activity was determined by evaluating each virus mean titer in treated and nontreated cells. The difference between both titers indicates the antiviral activity [28].

Statistical analysis

All experiments were conducted in triplicate ($n = 3$). All the values were represented as mean ± SD. Significant differences between the means of parameters as well as IC_{50} values were determined by probit analysis using the SPSS software program (SPSS Inc., Chicago, Illinois, USA).

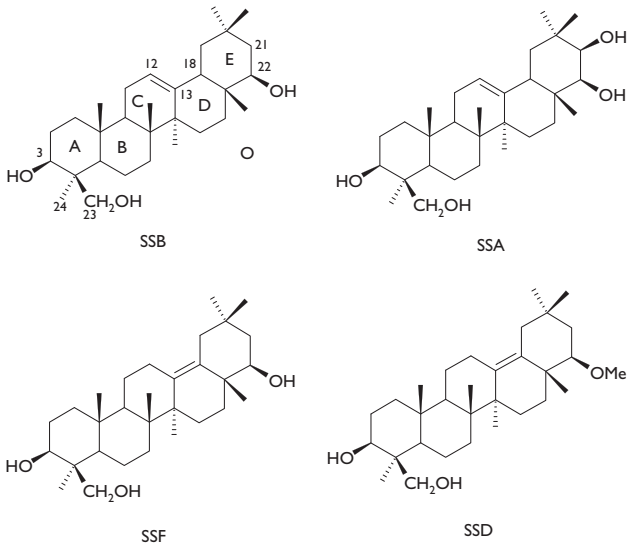
Results and discussion

In-vitro cytotoxic activity

Four soyasapogenols (SSA, SSB, SSD, and SSF; Fig. 1), were examined *in-vitro* for their cytotoxic activities against three human cancer cell lines (HCT-116, Hep-G2 and MCF-7) and one normal human cell line (HFB-4) using SRB assay. Their activities were compared with cytotoxicity of TSSE and doxorubicin, a positive control. The compounds examined were produced in our previous work by acid or enzymatic hydrolysis of the crude soybean saponin extract [6].

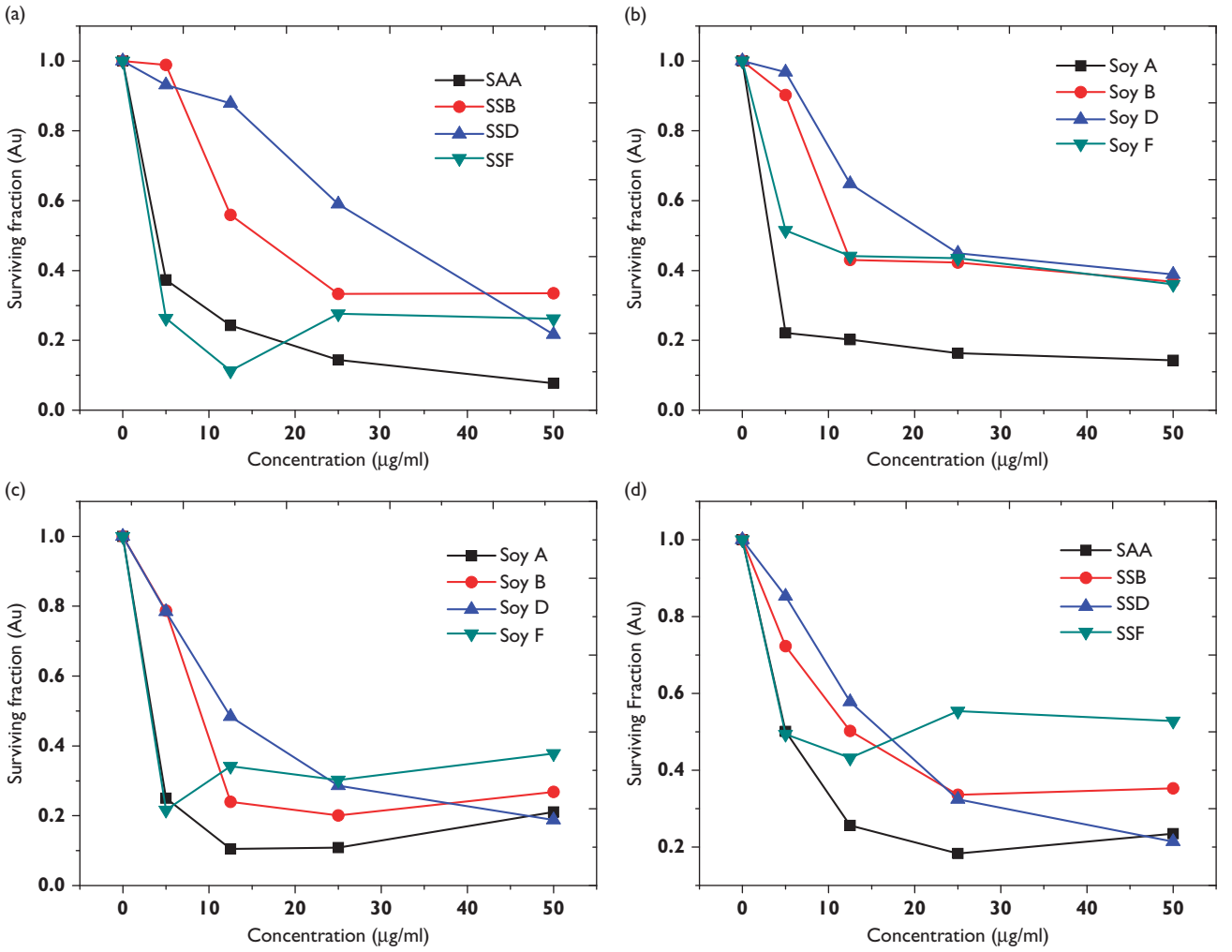
Results show that all tested soyasapogenols together with TSSE showed dose-dependent cytotoxic activities against four tested human cell lines (Figs 2 and 3). Cytotoxic activities, reflected by their IC_{50} values, against HCT-116 and Hep-G2 were in the following order: SSF > SSA > doxorubicin > SSB > SSD > TSSE, whereas, against MCF-7, they were in the following order: SSA = doxorubicin > SSF > SSB > SSD > TSSE. However, cytotoxic activities against HFB-4 were in the following order: SSF = SSA > doxorubicin > SSB > SSD > TSSE. The obtained IC_{50} values of SSA, SSB, and TSSE (3.89, 15.8, and 37.5 µg/ml, respectively) against Hep-G2 after 48 h were much lower than those reported by Zhang and Popovich [4] (50, 130, and 600 µg/ml, respectively) after 72 h. In terms of the cytotoxic activity against MCF-7, both

Figure 1



Structure of the investigated soyasapogenols. SSA, soyasapogenol A; SSB, soyasapogenol B; SSD, soyasapogenol D; SSF, soyasapogenol F.

Figure 2

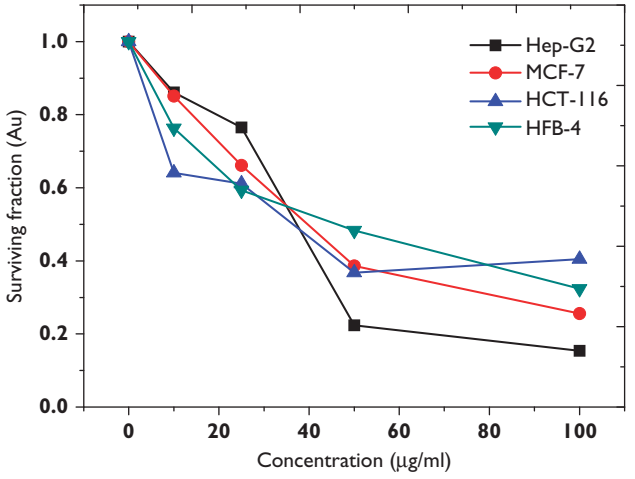


Cytotoxic activities of soyasapogenols (SSA, SSB, SSD and SSF) against (a) human liver carcinoma cell line (Hep-G2); (b) human breast carcinoma cell line (MCF-7); (c) human colon carcinoma cell line (HCT-116); (d) normal human melanocytes (HFB-4) using SRB assay. Values are mean ± SD of three separate experiments, each in triplicate.

SSA and SSB had potent cytotoxic effects after 48 h on MCF-7 cells with IC_{50} values of 2.97 µg/ml (6.27 µmol/l) and 11.4 µg/ml (24.89 µmol/l), respectively (Table 1). In contrast, Rowlands *et al.* [18] reported that SSA stimulated the proliferation of estrogen-sensitive cells MCF-7 2.5-fold; however, SSB exerted no significant effect on MCF-7 cells at all concentrations up to 10 µmol/l after 72 h [18]. It is worth noting that there are no previously reported data on the cytotoxic activity of TSSE against MCF-7; however, TSSE showed an IC_{50} value of 39.3 µg/ml after 48 h in this study.

Analysis of the results in Table 1 comparing the structure of the measured soyasapogenols (Fig. 1) showed that the hydroxyl groups at C-21 and C-22 play a major role in the activity of the measured compounds in addition to the double bond between C-12 and C-13 as well as C-13 and C-18. SSF, which has a β-hydroxyl group at C-22, has a good activity as compared with the positive control, doxorubicin. This activity decreased markedly on just replacing the β-hydroxyl group by a β-methoxyl group in SSD. However, this did not explain the decrease in the

Figure 3



Cytotoxic activities of total soyasaponin extract against human liver carcinoma cell line (Hep-G2), human breast carcinoma cell line (MCF-7), human colon carcinoma cell line (HCT-116), and normal human melanocytes (HFB-4) using the SRB assay. Values are mean ± SD of three separate experiments, each in triplicate.

Table 1 Cytotoxicity of soyasapogenols (SSA, SSB, SSD and SSF) and TSSE against HCT-116, Hep-G2, MCF-7 and HFB-4 cell lines as measured by 50% cell toxicity (IC_{50}) using SRB assay

	IC_{50} (μg/ml)			
Compounds	HCT-116	Hep-G2	MCF-7	HFB-4
SSA	3.12	3.89	2.97	5.1
SSB	8.76	15.8	11.4	12.6
SSD	11.8	30.7	21.4	16.5
SSF	3.0	3.12	6.0	5.1
TSSE	36.2	37.5	39.3	45.6
Doxorubicin (positive control)	3.73	4.0	2.97	8.0

HCT-116, human colon carcinoma cell line; Hep-G2, liver carcinoma cell line; HFB-4, normal human melanocytes; MCF-7, human breast carcinoma cell line; SRB, sulforhodamine B, TSSE, total soyasaponin extract.

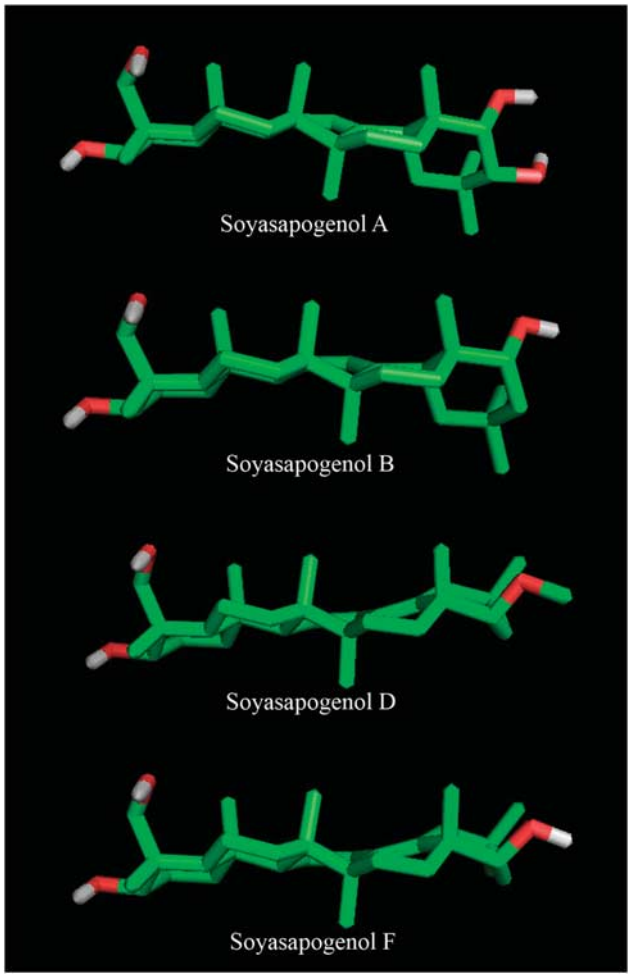
activity of SSB, which also has a β-hydroxyl group at C-22. The energetically optimized 3D structure of the measured compounds (Fig. 4) shows that both SSA and SSB have rings A, B, C, and D in the same plane and because of the presence of a double bond between C-12 and C-13, ring E adopts a position perpendicular to the molecular plane. Consequently, the β-hydroxyl group at C-21 is aligned with the molecular plane, whereas that at C-22 is perpendicular to it (Fig. 4). SSF, which has a double bond between C-13 and C-18, has rings A, B, C, D and E in the same plane; consequently, the β-hydroxyl group at C-22 is aligned with the molecular plane (Fig. 4). In conclusion, if the β-hydroxyl group at C-21 or C-22 was aligned with the plane of the molecule, a marked increase in the activity of the soyasapogenol was produced. These may be responsible for the good activity of both SSA and SSF compared with the other soyasapogenols and the positive control.

Moreover, all soyasapogenols showed very good cytotoxic activities against all cell lines compared with TSSE itself. These results are in agreement with previously reported data [3,30,31]. Gurfinkel and Rao [17] have reported that there was a relationship between structure and bioactivities, with SSA and SSB generally being more bioactive compared with their glycosides [17]. There is some evidence, as with many other saponins, that the bioactivity of soyasaponins increases as sugar moieties are eliminated from the saponin structure, thereby reducing the polarity [29]. Generally, the SSA-containing extract was found to show the greatest propensity to affect the cell cycle compared with the SSB-containing extract compared with a fractionated extract or a total saponin mixture [4,30]. To the best of our knowledge, there are no previously reported data on the cytotoxicity of SSD and SSF. However, there are some reports on the cytotoxicity and hepatoprotective effects of soyasaponins and SSA and SSB.

In-vitro antioxidant activity

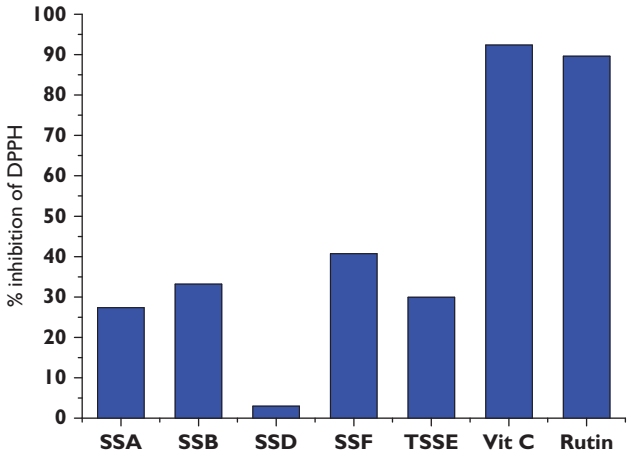
The antioxidant activity of four soyasapogenols and TSSE was evaluated using the DPPH radical-scavenging method. Results presented in Fig. 5 show that all soyasapogenols and TSSE did not show appreciable

Figure 4



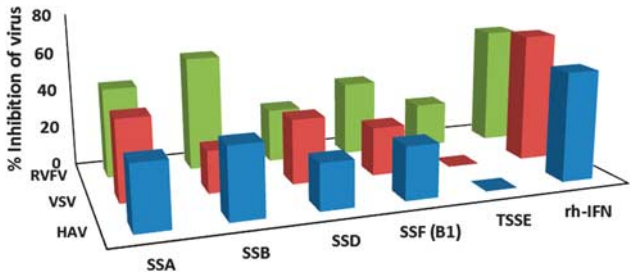
The energetically optimized three-dimensional structure of the measured soyasapogenols. Hydrogen atoms were deleted after energy minimization to clarify the plane of the compounds and the hydroxyl groups.

Figure 5



Antioxidant activities of soyasapogenols (SSA, SSB, SSD and SSF) and total soyasaponin extract (TSSE) using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical-scavenging assay. Values are mean ± SD of three separate experiments, each in triplicate.

Figure 6



Antiviral activity of soyasapogenols; SSA (25 μg/ml), SSB (25 μg/ml), SSD (25 μg/ml), SSF (12.5 μg/ml) and total soyasaponin extract (TSSE) (100 μg/ml) against three viruses: Rift Valley fever virus (RVFV), vesicular stomatitis virus (VSV) and hepatitis A virus (HAV). Recombinant human interferon α2a (rh-IFN α2a, 10 IU/0.1 ml) was used as a positive control. Values are mean ± SD of three separate experiments, each in triplicate.

scavenging activity compared with the standards (ascorbic acid and rutin), reflected by their DPPH inhibition percentage at a concentration of 100 μmol/l. The DPPH inhibition was in the following order: ascorbic acid > rutin > SSF > SSB > TSSE > SSA > SSD, where their DPPH inhibition percentages were: 92.35 > 89.62 > 40.72 > 33.19 > 29.9 > 27.32 > 3.09 (respectively). To the best of our knowledge, there are no previously reported data on the direct antioxidant activity of all tested compounds.

In-vitro antiviral activity

Researchers believe that saponins can stimulate the immune system, ward off microbial and fungal infections, protect against viruses and even act as a spermicide [31]. Therefore, the antiviral activity of the four soyasapogenols and TSSE was evaluated against three viruses (RVFV, VSV and HAV) using the highest nontoxic concentration for each compound. Figure 6 shows that all tested soyasapogenols had a significant antiviral activity against the three viruses, reflected by their high inhibition percentage of the log virus titer count. Their activities against RVFV and HAV viruses were in the following order: rh-IFN > SSB > SSA > SSF > SSD > TSSE. However, those against VSV were in the following order: rh-IFN > SSA > SSD > SSF > SSB > TSSE. Although the concentration of TSSE (100 μg/ml) used was greater than those used for SSB, SSA, SSD and SSF (25, 25, 25 and 12.5 μg/ml, respectively), it showed no activity against the VSV virus and an elevation in the HAV virus count (2.5%). Consequently, sugar moieties attached at the C-3 position and/or at the C-22 position of the aglycone seemed to eliminate or reduce its antiviral activity.

The chemical structure of the tested compounds also shows that the hydroxyl group and the double bond control the activity of the compounds. SSB showed the highest activity against both RVFV and HAV viruses. Analysis of the 3D structure (Fig. 4) shows that the presence of a β-hydroxyl group at C-22 in a position perpendicular to the plane of the molecule enhances the antiviral activity of the compound. However, SSA showed the maximum activity against the VSV virus. As SSA is a hydroxylated derivative of SSB at C-21, it might enhance the anti-VSV activity, whereas it may not be essential for the anti-RVFV and anti-HAV activities. In addition, SSD and SSF antiviral activities were comparable against the tested viruses. They showed better anti-VSV activities compared with SSB, indicating that the double bond in ring D may play a role in their anti-VSV activities. To the best of our knowledge, there are no previous reports on the antiviral activities of the tested soyasapogenols or TSSE against those three viruses (RVFV, HAV, and VSV).

Conclusion

Among the tested soyasapogenols, SSA and SSF showed the best therapeutic values against Hep-G2, HCT-116 and MCF-7 cell lines. Analysis of the 3D structure of these compounds indicated that if the β-hydroxyl group at C-21 or C-22 was aligned with the plane of the molecule, a marked increase in the cytotoxic activity of the soyasapogenol was produced. In terms of their antiviral activity against RVFV, VSV and HAV viruses, all soyasapogenols showed significant inhibition activities compared with TSSE itself. These results indicate that the hydroxylation at C-21 as well as the presence of a double bond in ring D instead of ring C might enhance anti-VSV activity, whereas it may not be essential for anti-RVFV and anti-HAV activities. However, the tested soyasapogenols and TSSE did not show appreciable antioxidant activity. These comparative data suggested that the investigated soyasapogenols could be candidate therapeutic agents as anticancer and antiviral agents. However, further studies may be required to examine the mode of action of each compound.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K. Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem 1995; 43:1184–1192.

2 Shi J, Arunasalam K, Yeung D, Kakuda Y, Mittal G, Jiang Y. Saponins from edible legumes: chemistry, processing, and health benefits. J Med Food 2004; 7:67–78.

3 Hu J, Zheng YL, Hyde W, Hendrich S, Murphy PA. Human fecal metabolism of soyasaponin I. J Agric Food Chem 2004; 52:2689–2696.

4 Zhang W, Popovich DG. Effect of soyasapogenol A and soyasapogenol B concentrated extracts on Hep-G2 cell proliferation and apoptosis. J Agric Food Chem 2008; 56:2603–2608.

5 Watanabe M, Mido N, Tamura T, Sumida N, Yaguchi T. Saponin digesting enzymes, genes thereof and soyasapogenol B mass production system. USA 2006; patent no.: 7,022,508 B2.

6 Amin HAS, Hanna AG, Mohamed SS. Comparative studies of acidic and enzymatic hydrolysis for production of soyasapogenols from soybean saponin. Biocatal Biotransform 2011; 29:311–319.

7 Hu XY, Wang XG. Prospect of soya-saponins (I): the distribution, structure and characteristics. Chin Oils Fats 2001; 26:29–33.

8 Kinjo J, Hirakawa T, Tsuchihashi R, Nagao T, Okawa M, Nohara T, Okabe H. Hepatoprotective constituents in plants 14. Effects of soyasapogenol B,

sophoradiol, and their glucuronides on the cytotoxicity of tert-butyl hydroperoxide to HepG2 cells. Biol Pharm Bull 2003; 26:1357–1360.

9 Kinjo J, Yokomizo K, Hirakawa T, Shii Y, Nohara T, Uyeda M. Anti-herpes virus activity of fabaceous triterpenoidal saponins. Biol Pharm Bull 2000; 23: 887–889.

10 Oh YJ, Sung M-K. Soybean saponins inhibit cell proliferation by suppressing PKC activation and induce differentiation of HT-29 human colon adenocarcinoma cells. Nutr Cancer 2001; 39:132–138.

11 Ishii Y, Tanizawa H. Effects of soyasaponins on lipid peroxidation through the secretion of thyroid hormones. Biol Pharm Bull 2006; 29:1759–1763.

12 Ahn KS, Kim JH, Oh SR, Min BS, Kinjo J, Lee HK. Effects of oleanane-type triterpenoids from fabaceous plants on the expression of ICAM-1. Biol Pharm Bull 2002; 25:1105–1107.

13 Ellington AA, Berhow M, Singletary KW. Induction of macroautophagy in human colon cancer cells by soybean B-group triterpenoid saponins. Carcinogenesis 2005; 26:159–167.

14 Xiao JX, Huang GQ, Zhang SH. Soyasaponins inhibit the proliferation of Hela cells by inducing apoptosis. Exp Toxicol Pathol 2007; 59:35–42.

15 Kanduc D, Mittelman A, Serpico R, Sinigaglia E, Sinha AA, Natale C, *et al*. Cell death: apoptosis versus necrosis (review). Int J Oncol 2002; 21: 165–170.

16 Berhow MA, Wagner ED, Vaughn SF, Plewa MJ. Characterization and antimutagenic activity of soybean saponins. Mutat Res 2000; 448:11–22.

17 Gurfinkel DM, Rao AV. Soyasaponins: the relationship between chemical structure and colon anticarcinogenic activity. Nutr Cancer 2003; 47: 24–33.

18 Rowlands JC, Berhow MA, Badger TM. Estrogenic and antiproliferative properties of soy sapogenols in human breast cancer cells *in-vitro*. Food Chem Toxicol 2002; 40:1767–1774.

19 Okubo K, Kudou S, Uchida T, Yoshiki Y, Yoshikoshi M, Tonomura M. Soybean saponin and isoflavonoids: structure and antiviral activity against human immunodeficiency virus *in-vitro*. Acs Symp Ser 1994; 546:330–339.

20 Li JB, Hu JS, Cheng BH, Wang XG, An ZY, Wei YD. Inhibitory effect of total soyasaponin on virus replication and its clinical application. Chin J Exp Clin Virol 1995; 9:111–114.

21 Ikeda T, Yokomizo K, Okawa M, Tsuchihashi R, Kinjo J, Nohara T, Uyeda M. Anti-herpes virus type 1 activity of oleanane-type triterpenoids. Biol Pharm Bull 2005; 28:1779–1781.

22 Hayashi K, Hayashi H, Hiraoka N, Ikeshiro Y. Inhibitory activity of soyasaponin II on virus replication *in-vitro*. Planta Med 1997; 63:102–105.

23 Yang X, Dong C, Ren G. Effect of soyasaponins-rich extract from soybean on acute alcohol-induced hepatotoxicity in mice. J Agric Food Chem 2011; 59:1138–1144.

24 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al*. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990; 82:1107–1112.

25 Gamez EJC, Luyengi L, Lee Sang Kook, Zhu L-F, Zhou B-N, Fong HHS, *et al*. Antioxidant flavonoid glycosides from daphniphyllum calycinum. J Nat Prod 1998; 61:706–708.

26 Liu L, Sun Y, Laura T, Liang X, Ye H, Zeng X. Determination of polyphenolic content and antioxidant activity of kudingcha made from Ilex kudingcha C.J. Tseng. Food Chem 2009; 112:35–41.

27 Specter SC, Hodinka RL, Weidbrauk DL, Young SA. *Clinical virology manual*. 4th ed. ASM Press; 2009.

28 Finter NB. Dye uptake methods for assessing viral cytopathogenicity and their application to interferon assays. J Gen Virol 1969; 5:419–427.

29 Shin HJ, Cho MS, Jung SY, Kim HI, Im KI. *In-vitro* cytotoxicity of *Acanthamoeba* spp. isolated from contact lens containers in Korea by crystal violet staining and LDH release assay. Korean J Parasitol 2000; 38:99–102.

30 Zhang W, Yeo MC, Tang FY, Popovich DG. Bioactive responses of Hep-G2 cells to soyasaponin extracts differs with respect to extraction conditions. Food Chem Toxicol 2009; 47:2202–2208.

31 Yu D, Morris-Natschke SL, Lee KH. New developments in natural products-based anti-AIDS research. Med Res Rev 2007; 27:108–132.