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# Biologically active steroidal glycosides from *Tribulus terrestris*

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The steroidal saponin constituents obtained from *Tribulus terrestris* were tested for their antimicrobial and cytotoxic effects. The spirostanol-based steroidal saponins **1–3** exhibited remarkable activity against fungal organisms (*Candida albicans* and *Cryptococcus neoformans*) and cancer cell lines [human malignant melanoma (SK-MEL), human oral epidermoid carcinoma (KB), human breast ductal carcinoma (BT-549), and human ovary carcinoma (SK-OV-3)], while none of the compounds possessing the furostanol framework **4–7** showed activity. The most active spirostanol glycoside, compound **3** exhibited a broad range of anticancer activity against cell lines, SK-MEL, KB, BT-549 and SK-OV-3 at $IC_{50}$s of 6.0, 7.0, 6.0 and 8.2 μg/ml, respectively, while compounds **1** and **2** showed selective cytotoxicity against SK-MEL at 6.7 and 9.1 μg/ml, respectively. The minimum inhibitory concentrations (MIC) in antifungal bioassay for compounds **1–3** varied from 1.5 to 6.2 μg/ml, which prompted to conclude certain structural features are required for these bioactivities.

## 1. Introduction

*Tribulus terrestris* Linn. (Zygophyllaceae), is found growing in subtropical areas around the world. The ethnopharmacological claims for *T. terrestris* include the use of decoctions or infusions in cases of spermatorrhea, phosphaturia, and disease of the genitourinary system, as well as kidney, liver and eye diseases in ancient Ayurvedic and Chinese traditional medicines [1, 2]. The earlier investigations performed on *Tribulus* species resulted in the isolation of steroidal saponins, lignanamides, alkaloids and flavonoids [3–9]. As a part of our ongoing study of various medicinal plant, and their active constituents, we initiated a phytochemical study of *T. terrestris* that have led into isolation of seven known steroidal-type glycosides. Since steroidal glycosides constitute an important class of secondary metabolites, known to exhibit antifungal and anticancer activities [10–14], an attempt has been made to determine the antimicrobial and cytotoxic effects of steroidal glycosides isolated from *T. terrestris*.

## 2. Investigations, results and discussion

All the compounds tested (**1–7**) were inactive against *S. aureus*, MRS, *A. fumigatus*, *P. aeruginosa*, and *M. intracellulare*. Although steroidal glycosides tested were from the same chemical class, only compounds **2** and **3** exhibited significant antifungal activity against *C. albicans* and *C. neoformans*, shown in Table 1. These results indicate that there are critical structural features responsible for antifungal activity. While the furostanol-type steroidal glycosides were inactive, two of the spirostanol glycosides (**2** and **3**) were significantly active, confirming earlier observations [10]. In contrast, **1** unexpectedly lacked activity.

**Table 1: Antifungal activity of compounds 1–7 (μg/ml)**

| Compounds | *Candida albicans* | | *Cryptococcus neoformans* | |
|---|---|---|---|---|
| | IC 50 | MIC | IC 50 | MIC |
| **1** | >50 | >50 | >50 | >50 |
| **2** | 3.50 | 6.25 | 2.00 | 3.12 |
| **3** | 2.50 | 6.25 | 0.70 | 1.56 |
| **4–7** | >50 | >50 | >50 | >50 |
| Amphotericin B | 0.04 | 0.08 | 0.30 | 0.62 |

**Table 2: Cytotoxic activity of compounds 1–7 (IC 50, μg/ml)**

| Compounds | SK-MEL | KB | BT-549 | SK-OV-3 | VERO |
|---|---|---|---|---|---|
| **1** | 6.7 | >50 | >50 | >50 | >50 |
| **2** | 9.1 | >50 | >50 | >50 | >50 |
| **3** | 6.0 | 7.0 | 6.0 | 8.2 | 7.0 |
| **4–7** | >50 | >50 | >50 | >50 | >50 |

The chemical difference between the aglycons of **2**, **3,** and **1** were the presence of a carbonyl group at C-12 for **2** and **3**, and the configuration of C-5 which was β for **1** (*cis*-decalin) and α for **2** and **3** (*trans*-decalin).

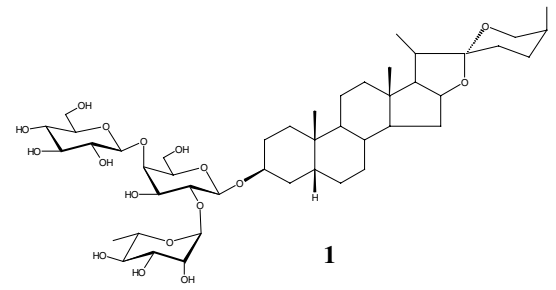
The cytotoxic activity of compounds **1–7** were evaluated against SK-MEL, KB, BT-549, SK-OV-3 cancer cell lines, and VERO normal cell line. Only spirostanol-type steroidal glycosides exhibited cytotoxicity, listed in Table 2. These results indicate that the spirostanol framework is essential to exert cytotoxic activity. Among the active compounds, **3** was found active against all cancer cell lines, while **1** and **2** were found active against only SK-MEL cells. Furthermore, spirostanol pentaglycoside **3**, showed wider spectrum and more potency than other two spirostanol tri-, and tetraglycosides **1** and **2**, respectively. Based on the results, steroidal framework and oligosaccharide residue attached at C-3 of aglycon, seem very important for both antifungal and cytotoxic effects of these steroidal glycosides.

Further studies are required to confirm the above assumptions regarding structure-activity relationship of spirostanol-type steroidal glycosides.

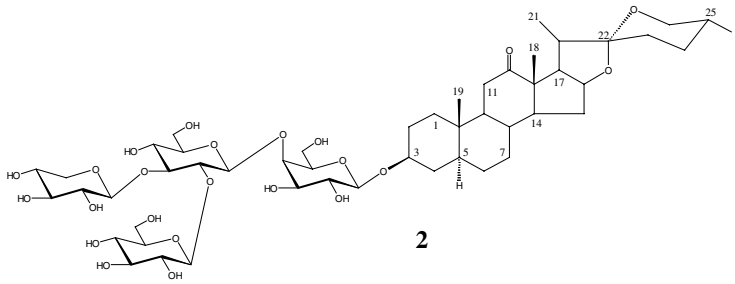
## 3. Experimental

### *3.1. Materials*

Steroidal glycosides **1–7** were isolated from the following plant materials: Compounds **1** {25(*S*)-5β-spirostan-3β-ol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside}, **4** {26-*O*-β-D-glucopyranosyl-(25*S*)-5β-furost-20(22)-en-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside} and **5** {26-*O*-β-D-glucopyranosyl-(25*S*)-5β-furost-20(22)-en-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside} from Chinese *T. terrestris* fruits [14], compounds **2**, {25(*R*)-5α-spirostan-3β-ol-12-on-3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} [15], **3** {25(*R*)-5α-spirostan-3β-ol-12-on-3-*O*-β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside} [16] and **7** {25(*R*)-5α-furostan-22-



**1**



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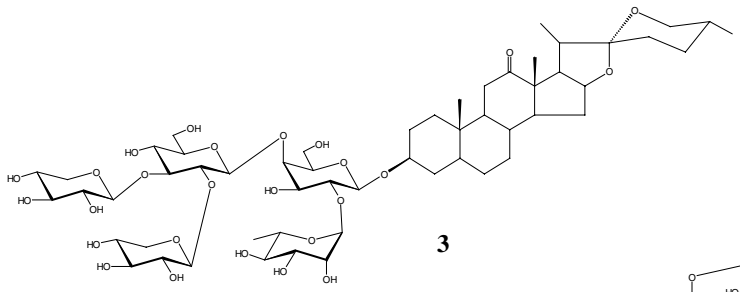

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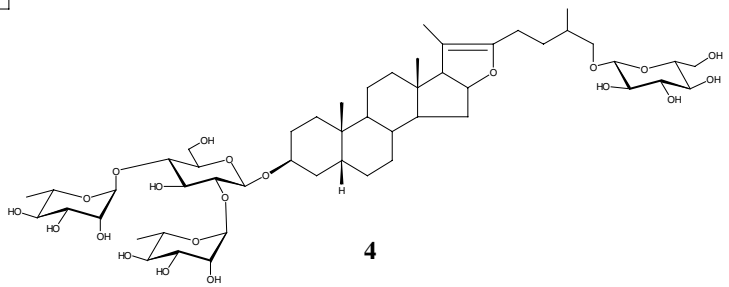

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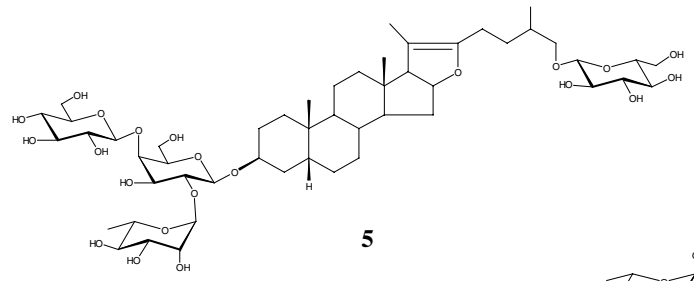
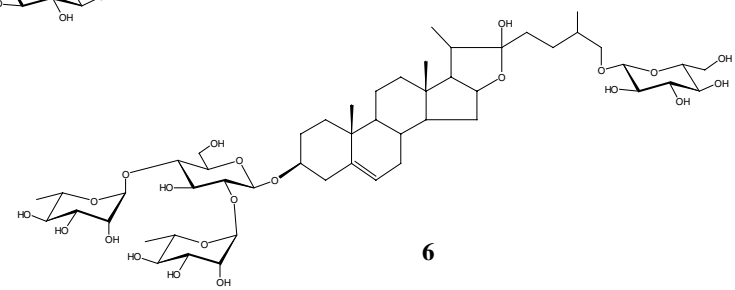


**5**



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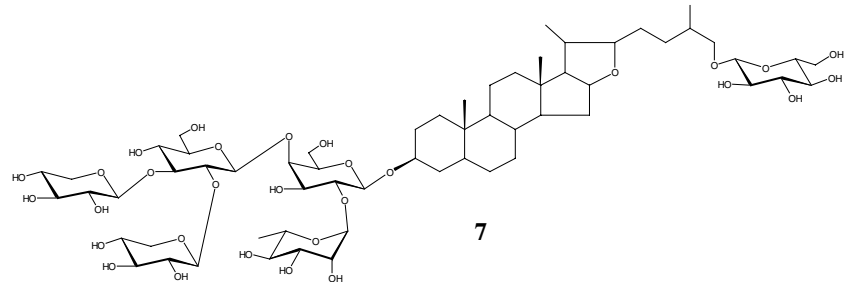

**7**

methoxy-3β,-diol-26-*O*-β-D-glucopyranosyl-3-*O*-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-galactopyranoside}[17] from Indian *T. terrestris* stems, and **6** {25(*R*)-furost-5-ene-3β,-diol-26-*O*-β-D-glucopyranosyl-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside [11] from Bulgarian *T. terrestris* fruits (These samples were provided by USA NutraSource, Inc., 1300 Industrial Road Unit 16, San Carlos, CA 94070). All compounds were identified based on spectral data (IR, HRESIMS, 1D-, and 2D-NMR).

### *3.2. Antimicrobial bioassay*

Fungal organisms employed in the OIGM assay include: *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906. Bacteria include *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 43300 (MRS), *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. All organisms are stored on agar slants at 4 °C until needed [*C. albicans* and *C. neoformans* on Sabouraud Dextrose agar (Difco, Detroit), *S. aur-*

*eus*, MRS and *P. aeruginosa* on Eugon agar (Difco, Detroit), *M. intracellulare* on Lowenstein-Jensen agar (BBL, Maryland), and *A. fumigatus* on YM agar]. Susceptibility testing is performed using a modified version of the NCCLS methods (1). Excluding *A. fumigatus* (which is prepared on the day of the assay), all microorganisms are subcultured prior to the assay by suspending cells from the slant in the appropriate broth and incubating at varying temperatures and times: *C. albicans* in Sabouraud Dextrose broth (Difco, Detroit) for 24 h at 37 °C, *C. neoformans* in Sabouraud Dextrose broth for 72 h at 30 °C, *S. aureus* and MRS in Eugon broth (BBL, Maryland) for 24 h at 37 °C, *M. intracelluare* in Middlebrook broth with OADC enrichment (BBL, Maryland) for 72 h at 37 °C, *P. aeruginosa* in Eugon broth for 6 h at 37 °C. For the assay, the microbial inocula, excluding *A. fumigatus*, are prepared by diluting the subcultured organism in its incubation broth: *S. aureus* and MRS = 1 to 50 dilution, *P. aeruginosa* = 1 to 1000 dilution, *C. albicans* = $1 \times 10^4$ cells/ml determined by a hemacytometer count of the saline-washed overnight culture, *C. neoformans* = $2 \times 10^5$ cells/ml determined turbidimetrically, *M. intracellulare* = 1 to 30 dilution. The *A. fumigatus* inoculum is prepared by gently removing the growth from a slant and transferring to 50 ml YPD broth. Prepared test compounds/extracts are dissolved in DMSO, serially-diluted using normal saline, and transferred in duplicate to 96 well microtiter plates (flat bottom plates for *C. albicans*, *C. neoformans*, *S. aureus*, MRS and *P. aeruginosa* and round bottom plates for *M. intracelluare* and *A. fumigatus*). The microbial inoculum is added to achieve a final volume of 200 µl and final concentrations starting with 500 µg/ml for crude extracts and 50 µg/ml for pure compounds. Drug [tetracycline (Sigma, St. Louis) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi] as well as growth and blank (media only) controls are added to each test plate. Except for *M. intracellulare* and *A. fumigatus*, which are inspected visually, all other organisms are read turbidimetrically at 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) prior to and after incubation: *C. albicans*, *S. aureus*, MRS and *P. aeruginosa* at 37 °C for 24 h, *C. neoformans* and *A. fumigatus* at 30 °C for 72 h, and *M. intracellulare* at 37 °C for 72 h). For turbidimetrically-read organisms, percent growth is calculated and plotted versus concentration to afford the $IC_{50}$/MIC. Minimum fungicidal or bactericidal concentrations (MFC/MBC) are determined by removing 5 µl of each duplicate, transferring to agar and incubating at previously-mentioned times and temperatures. The MFC/MBC is defined as the lowest concentration of sample to allow no growth [17,18].

### *3.3. Anti-cancer assay*

Compounds were evaluated for anti-cancer activity against four cancer cell lines: SK-MEL, KB, BT-549, and SK-OV-3. Neutral red staining was used to determine the number of viable cells after treatment with compounds. The anti-cancer assay is run at two different levels: i.) A primary assay for initial natural product screening at a single concentration, and ii.) a secondary assay for retesting active substances at three to six concentrations. The anti-cancer results are compared to results from normal mammalian VERO cell testing with the same substances in order to determine level of cytotoxicity. The assay is based on the fact that viable cells attached to bottom of the plate will accumulate neutral red dye in their lysosomes, and it will remain inside the cell while the liquid supernatant is washed away with warm saline. A subsequent addition of 2-propanol will lyse the cells releasing the dye into solution; hence, a higher number of viable cells in a well will yield a greater absorbance and an increase in reddish hue. *Day 1*: 25,000 cells are seeded into each well of a culture-treated 96-well microtiter plate and allowed to incubate for 24 h. *Day 2*: Diluted test samples are added in duplicate to the plate. Final test concentrations are 10, 3.3, and 1.1 µg/ml for pure compounds, while extracts are initially tested at 100 µg/ml, and then follow-up secondary testing of active extracts is performed at 100, 33, and 11 µg/ml. The plates are then allowed to incubate 48 h. *Day 4*: Media is dumped from the plates, and neutral red dye is added. After 90 min of incubation, the excess dye is dumped and each well is washed with warm saline. The cells are then lysed by adding a 0.33% HCl in isopropanol solution. Absorbance is then measured at dual wavelength settings of 490 nm and 630 nm. Corresponding growth inhibition numbers are calculated and graphed. For secondary assays, $IC_{50}$'s are determined from logarithmic graphs of growth inhibition values [15].

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