

Anti-*Trichomonas* activity of *Sapindus* saponins, a candidate for development as microbicidal contraceptive

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Objectives: Trichomoniasis is the most common non-viral sexually transmitted disease and is caused by the protozoan *Trichomonas vaginalis*. In view of increased resistance of the parasite to classical drugs of the metronidazole family, the need for new unrelated agents is increasing. This study evaluates anti-*Trichomonas* activity of *Sapindus* saponins, a component of a herbal local contraceptive *Consap* recently marketed in India.

Methods: The parasites were treated with saponins for MIC determination. Anti-*Trichomonas* activity of the saponins was evaluated using a cytoadherence assay, the substrate gel electrophoresis method and RT-PCR analysis. The effect of saponins on the mitochondrial potential of the host was determined by fluorescence-activated cell sorter. Actin cytoskeletal staining was used to determine the effect on parasite cytoskeleton.

Results: Using *in vitro* susceptibility assay, the MIC of *Sapindus* saponins for *T. vaginalis* (0.005%) was found to be 10-fold lower than its effective spermicidal concentration (0.05%). Saponins concentration dependently inhibited the ability of parasites to adhere to HeLa cells and decreased proteolytic activity of the parasite's cysteine proteinases. This was associated with decreased expression of adhesin AP65 and membrane-expressed cysteine proteinase TvCP2 genes. Saponins produced no adverse effect on host cells in mitochondrial reduction potential measurement assay. Saponins also reversed the inhibitory mechanisms exerted by *Trichomonas* for evading host immunity. Early response of saponins to disrupt actin cytoskeleton in comparison with their effect on the nucleus suggests a membrane-mediated mode of action rather than via induction of apoptosis.

Conclusions: Findings demonstrate the potential of *Sapindus* saponins for development as a microbicidal contraceptive for human use. Further studies are required to evaluate its microbicidal activity against other sexually transmitted infections.

Keywords: susceptibility, cytoadherence, adhesin, *Consap*

Introduction

Trichomoniasis, caused by the protozoan *Trichomonas vaginalis*, is one of the most prevalent non-viral human urogenital infections with ~180 million people around the world suffering from this disease.¹ Trichomoniasis is associated with many perinatal complications and male and female genitourinary tract infection, and has been linked with cervical cancer,²⁻⁴ atypical pelvic inflammatory disease⁵ and infertility.^{6,7} It is also believed to act as a co-factor in HIV transmission and acquisition.⁸ Women who are infected with this parasite during pregnancy are

predisposed to premature rupture of placental membranes, premature labour and low-birth-weight infants.^{9,10} This disease thus has crucial medical and socio-economic implications.

Proteins and glycoproteins on the cell surface of *Trichomonas* play a major role in their adhesion, nutrient acquisition and host-parasite interaction. Adhesion of trichomonads to the vaginal epithelial cells is a critical step in their virulence and pathogenesis.¹¹ This adhesion is mediated primarily by four adhesion proteins, AP65, AP51, AP33 and AP23, acting in specific receptor-ligand fashion and is time-, temperature- and pH-dependent.¹² In addition, the role of cysteine proteinases (CPs) for

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adherence of *T. vaginalis* to vaginal epithelial cells has been suggested.¹³ Pre-treating trichomonads with *N*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), a specific CP inhibitor, causes a marked decline in their ability to adhere to epithelial cells, which is restored following addition of CPs to TLCK-treated cells.¹³ Since *T. vaginalis* lacks the ability to synthesize lipids, erythrocytes serve as the prime source of fatty acids needed by the parasite. In addition to lipids, iron is another important nutrient for *T. vaginalis* and is acquired via lysis of erythrocytes.¹⁴ *T. vaginalis* has between 11 and 23 distinct CP activities, most of which are lysosomal^{13,15,16} and have been implicated as probable lytic factors in the haemolysis of erythrocytes.^{17–19}

The classic treatment for trichomoniasis involves drugs of the 5'-nitroimidazole family, of which metronidazole and tinidazole are the only effective approved drugs. Metronidazole has been the drug of choice for *T. vaginalis* infection. However, there has been an increase in the recognition of metronidazole-resistant trichomoniasis. Side effects from metronidazole treatment are common, and nausea and dizziness have been reported in up to 12% of the patients.^{20,21} Hypersensitivity reactions, usually manifesting as dermatological symptoms, can occur. Saponins have long been known to have cell membrane lytic and detergent action, and this is believed to be the result of affinity of the aglycone moiety for membrane sterols, particularly cholesterol, with which they form insoluble complexes.²² Saponins have also been found to facilitate changes in membrane fluidity, which results in alterations in enzyme activity of biological membranes and ion transport across them.²³ When binding with cholesterol, saponins change the lipid environment of membrane proteins, including ion channels, transporters and receptors. Owing to this, it has been suggested that they may cause secondary biochemical responses.²⁴

The *Sapindus* saponins investigated in this study are a mixture of six sapindosides (sapindosides A, B, C, D and mukurozisaponins E1 and Y1), with sapindoside B as one of the major constituents, isolated by *n*-butanol extraction of the ethanolic extract of fruit pericarp of *Sapindus mukorossi* Gaertn (Reetha or soap nut; family Sapindaceae) and identified by liquid chromatography-mass spectrometry.^{25,26} These saponins constitute a component of the herbal local contraceptive *Consap* recently marketed in India by M/s Hindustan Latex Ltd, Thiruvananthapuram and exhibit *in vitro* spermicidal activity at a concentration of 0.05% (i.e. 500 mg/L).^{27,28} The spermicidal action of these saponins is associated with the β -amyrin C-28 carboxylic acid type of saponin linked to a particular sequence of sugar moieties.²⁸ Previous studies from this institute show that repeated intravaginal application of *Consap* in rabbits (2.5%, 5% or 10% for 60 days) or rhesus monkeys (5%, 10% and 25% for 90 days) does not cause any local irritation/pathological lesions in the vagina or other parts of the genital tract, and no saponins could be detected in the blood, suggesting that these are not absorbed systemically (G. N. Srivastava, N. Sethi, A. K. Roy and S. K. Mukherjee, unpublished results). The present study demonstrates anti-*Trichomonas* activity of this *Sapindus* saponin mixture at a 10-fold lower concentration (0.005%) than its minimal effective spermicidal concentration.^{27,28} This study also demonstrates that *Sapindus* saponins disrupt the actin cytoskeleton network beneath the cell membrane and affect membrane-mediated adherence of *Trichomonas* to the host cells.

Materials and methods

Chemicals and materials

The *T. vaginalis* culture was a gift from the Post-Graduate Institute of Medical Education and Research, Chandigarh, India. The study was approved by the Institutional Ethics Committee. A purified sample of *Sapindus* saponins^{25–27} for this study was obtained from the Pharmaceuticals Division of the Institute. Metronidazole, TYI-S33 culture medium, fetal calf serum, vitamin mixture, penicillin-streptomycin mixture, JC-1 (1,1',3,3'-tetraethyl benzimidazole carbocyanines iodine), CCCP-1 (carbonyl cyanide *m*-chlorophenyl-hydrazone), proteinase K and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. TLCK, a specific CP inhibitor, was purchased from ICN Biochemicals, USA. Dilutions of the test agents were made in DMSO such that its final concentration in the working culture medium did not exceed 0.01%. Cultures treated with 0.01% DMSO served as vehicle control.

T. vaginalis culture

The parasites were grown in standard TYI-S33 medium (pH 6.8) supplemented with 10% FCS, vitamin mixture and 100 U/mL penicillin/streptomycin mixture at 37°C in 15 mL screw-stoppered glass tubes.²⁹ The cultures routinely attained a concentration of 2×10^7 cells/mL in 48 h. An inoculum of 1×10^4 cells per tube was used for maintenance of the culture.

Host cell line culture

The HeLa cervical epithelial cell line was purchased from the National Centre for Cell Sciences, Pune, India and the RAW264.7 murine monocyte/macrophage cell line TIB-71 from the American Type Culture Collection (ATCC), USA. These were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with NaHCO₃ (according to manufacturer's protocol), 100 U/mL penicillin/streptomycin mixture and 10% FCS (Gibco Invitrogen Corp., USA) at 37°C under 5% CO₂.

Susceptibility assay

Susceptibility of *T. vaginalis* was tested as described.³⁰ Briefly, 5×10^3 *Trichomonas* trophozoites per mL were incubated in the presence of serially diluted metronidazole (1–12 μ M) and *Sapindus* saponins (0.0001% to 0.02%) in the TYI-S33 culture medium in 24-well culture plates at 37°C. Cells were checked for viability at different time intervals from 3 to 48 h under the microscope at 20 \times magnification. Viability of the cells was determined by Trypan Blue exclusion assay. Cells were counted using a haemocytometer. Minimum concentration of the test agent at which all cells were found dead was considered as its MIC. Metronidazole (Sigma-Aldrich), the most popular drug presently available in the market, with an MIC of 6 μ M for *T. vaginalis* was used as reference standard.³¹ The experiment was repeated three times to confirm the MIC.

Cytoadherence assay

Measurement of the effect of saponins on the extent of *T. vaginalis* cytoadherence to HeLa cells was carried out by slight modifications of the previously standardized procedure.¹³ Trichomonads were labelled overnight with [³H]-thymidine in TYI-S33 medium. After 14 h of incubation, the cells were thoroughly washed with normal

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TYI-S33 medium to remove unincorporated [³H]-thymidine. Radiolabelled trichomonads were then treated with the saponins (0.002%, 0.005% and 0.01%) for 1 h at 37°C. Trophozoites treated with the specific proteinase inhibitor TLCK at 0.036%¹³ were used as negative controls, while labelled cells treated with 0.01% DMSO were considered as vehicle controls. Trichomonads were then washed twice with TYI-S33 medium without serum supplementation by centrifuging at 2500 g for 7 min each time, and the cells were resuspended in interaction medium [TYI-S33:DMEM (1:2) without serum supplementation]. For evaluation of inhibition of cytoadherence, 1 × 10⁶ [³H]-thymidine-labelled trichomonad trophozoites treated with the saponins or the vehicle (0.01% DMSO) were allowed to interact with 2 × 10⁵ HeLa cells for 30 min at 37°C. Cells were then thoroughly washed with DMEM-TYI-S33 (2:1) interaction medium for removal of non-adhered *Trichomonas* cells. HeLa cells were then dislodged and mixed (1:9) with the aqueous counting scintillation fluid (ACS II; Amersham, USA), and radioactivity due to [³H]-thymidine was measured on an LS Analyzer 6500 (Beckman Instruments Inc., USA).

CP activity

Substrate gel electrophoresis³² for the estimation of the activity of CPs of *Trichomonas* was used with slight modifications. Briefly, 1 × 10⁶ *T. vaginalis* trophozoites were incubated with the saponins (0.005%) or the vehicle (0.01% DMSO) for 2, 4 and 6 h in TYI-S33 medium at 37°C. The cells were pelleted and washed with sterile PBS (pH 7.4) by centrifuging at 2500 g for 6 min at 4°C. The pellet was suspended in lysis buffer (50 mM Tris HCl, 0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂ and 5 mM EDTA; pH 7.5). The cells were lysed by conventional freezing at -70°C and thawing at 30°C. Lysates were subjected to SDS-PAGE on 10% acrylamide gel co-polymerized with gelatin (final concentration: 0.2%, w/v). Electrophoresis was carried out at 100 V until the tracking dye reached the end of the plate. The gel was then incubated in 2.5% Triton-X 100 to remove SDS and allow proteinases to become active. Activity of proteinases was estimated by development of bands by immersing the gel in incubation buffer [0.1 M acetate buffer, pH 4.5, containing 1 mM dithiothreitol; Sigma-Aldrich] for 4 h at room temperature. The bands were visualized by staining in 0.25% (w/v) Coomassie Brilliant Blue and destaining in 40% methanol and 10% glacial acetic acid solution. Trophozoites incubated for 1 h with 1 mM TLCK (MP Biomedicals, USA) were used for comparison.

Mitochondrial reduction potential measurement assay

The effect of saponins on mitochondrial reduction potential of HeLa cells was assessed using JC-1, a mitochondria-specific cationic dye having dual fluorescence. HeLa cells were incubated with the saponins (0.005%) for 3 h and JC-1 (2 μM) was added during the last 10 min of incubation. Cells were washed, extracted from culture dishes and the fluorescence was read on a fluorescence-activated cell sorter with excitation at 490 nm. Cells exposed to CCCP-1 (100 μM), a known apoptosis inducer, for 1 h were used as positive controls for apoptosis.

MTT assay

The cytotoxic effect of saponins on host (HeLa) cells was evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Nonoxynol-9, the marketed local contraceptive, was used for comparison. 5 × 10⁵ HeLa cells grown in DMEM

supplemented with 10% FCS at 37°C under 5% CO₂ were treated with different concentrations of the saponins for 24 h. HeLa cells treated with 0.01% DMSO were taken as vehicle controls. After 24 h, 10 μL of MTT solution (5 mg/mL, pH 7.4) in fresh culture medium was added, and incubation was continued for 4–6 h at 37°C under 5% CO₂. The medium was then aspirated and formazone granules formed in the cells were dissolved in 100 μL of DMSO. The intensity of blue colour thus obtained was read at 530 nm on an ELISA reader (Versa Max, Molecular Devices, CA, USA).

Inflammatory response

Macrophages are important components of the innate immune system and are capable of producing pro-inflammatory cytokines such as TNF-α and IL-12p40. Inflammatory response of the host against *T. vaginalis* in the presence of *Sapindus* saponins (0.005%) was determined using murine macrophage monocytic cell line RAW264.7. The cells were interacted with *T. vaginalis* in the ratio of 1:10 in the presence of *Sapindus* saponins, or the vehicle and cytokine expression at 1, 4 and 7 h was evaluated by RT-PCR.

Reverse transcriptase-polymerase chain reaction

RT-PCR was carried out to examine mRNA expression levels of TNF-α and IL-12p40 in RAW264.7 cells at different time intervals of their interaction with *T. vaginalis* parasites in the presence of saponins. The samples were stored at -80°C until use. Total RNA was isolated with a TRIzol reagent (Invitrogen Life Technologies, USA) and reverse-transcribed using a cDNA synthesis kit (Ambion, Austin, USA) following measurement and normalization of total RNA concentration. The RNA sample was treated with 2 μL (concentration 1 U/μL) of DNase (Invitrogen Life Technologies) to remove any DNA contamination. The DNase was inactivated by incubation at 70°C for 10 min. A PCR was performed with gene-specific primer sets for AP65, TvCP2, TvCP12 and β-tubulin (*T. vaginalis*) and TNF-α, IL-12p40 and β-actin (murine) genes. For AP65, 25 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min) and extension (72°C for 2 min), for TvCP2 and TvCP12, 25 cycles of denaturation (94°C for 1 min), annealing (67.5°C for 1 min) and extension (72°C for 2 min), for β-tubulin, 25 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 2 min), for TNF-α and IL-12p40, 25 cycles of denaturation (94°C for 1 min), annealing (65°C for 1 min) and extension (72°C for 2 min) and for β-actin, 23 cycles of denaturation (94°C for 1 min), annealing (65°C for 1 min) and extension (72°C for 2 min) were used. Primer sequences and PCR product sizes for AP65, 5'-AAC GTG ACC GCC TTA ACC T-3' (sense), 5'-CGT GGT CTT CCT TCT TAA CA-3' (antisense), 650 bp; TvCP2, 5'-GAG ACA GGC AAC TTC TTC ACA-3' (sense), 5'-GTA GTC GAT AGC CTT GGA TG-3' (antisense), 450 bp; for TvCP12, 5'-GAT TTC AAC TTG CTT CCG GCA TT-3' (sense), 5'-GTT GAC TGT TTG GCC CTT GGA AA-3' (antisense), 270 bp; for β-tubulin, 5'-CAT TGA TAA CGA AGC TCT TTA CGA T-3' (sense), 5'-GCA TGT TGT GCC GGA CAT AAC CAT-3' (antisense), 300 bp; for TNF-α, 5'-ATG AGC ACA GAA AGC ATG ATC-3' (sense), 5'-TAC AGG CTT GTC ACT CGA ATT-3' (antisense), 276 bp; for IL-12p40, 5'-ATC TCC TGG TTT GCC ATC GTT TTG-3' (sense), 5'-TCC CTT TGG TCC AGT GTC ACC TTC-3' (antisense), 527 bp and for β-actin, 5'-TGT GAT GGT GGG AAT GGG TCA-3' (sense), 5'-TTT GAT GTC ACG CAC GAT TTC C-3' (anti-sense), 514 bp. Amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA).

PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining.

Phalloidin staining

For phalloidin staining, *T. vaginalis* cells treated with the saponins (0.005%) or the vehicle (0.01% DMSO) for 3 and 5 h were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature and their smear was prepared on 0.1% poly L-lysine-coated coverslips. After washing with PBS, coverslips were submerged in 0.1% Triton-X 100 in PBS for 10 min at room temperature for permeabilization of cells. After thorough washing with PBS, coverslips were covered with 1% BSA solution in PBS for 1 h at room temperature followed by three washings with PBS (pH 7.4). Cells were then treated overnight with phalloidin-fluorescein isothiocyanate at 4°C, washed and the coverslips were mounted on glass slides with 90% glycerol and observed under Leica DMLB Fluorescence microscope fitted with a Leica DC300 camera.

Statistical analysis

All experiments were performed at least three times. The data were analysed by the Student's *t*-test.

Results

Motility and viability of *Trichomonas*

In our initial observations, no growth was observed after 24 and 48 h of incubation at 0.005% concentration of saponins. Subsequently, it was observed that almost all cells were dead at 12 h of incubation at this concentration. However, at lower concentrations of 0.0001% and 0.0005%, the cells were viable even after 48 h of incubation. Viability and number of *Trichomonas* trophozoites were reduced by *Sapindus* saponins at 0.001% and 0.0025% concentrations with no live parasites at 0.005% concentration (MIC) of saponins at 12 h (Table 1). This was 10-fold lower than its effective spermicidal concentration (0.05%) against human spermatozoa. A change in morphology from oval

to a more rounded shape as in the case of pseudocysts (living cells but without apparent mobility) or dead parasites was also observed. In comparison, all the cells were found dead 24 h after incubation with the 6 µM (MIC against *T. vaginalis*) of metronidazole,³⁸ although cell number was greatly reduced at 6 h time period at 6 and 12 µM concentrations (Table 1).

Cytoadherence and CPs

[³H]-thymidine-labelled *T. vaginalis* parasites on treatment with saponins (0.005%) for 3 h showed ~46% reduction in cytoadherence to host cells in comparison to cells of the corresponding vehicle control group [Figure 1a and b; a colour version of this figure is available as Supplementary data at JAC online (<http://jac.oxfordjournals.org/>)]. The inhibition (~74%) observed after treatment with TLCK (0.036%), the specific CP inhibitor, was comparable to the inhibition (~61%; *P* > 0.05) observed at 0.01% concentration, which was 5-fold lower than the spermicidal concentration of saponins. At lower concentration of 0.002% of saponins, ~22% reduction in cytoadherence was observed. In comparison, TLCK (0.036%) completely abolished proteolytic activity of CPs of the parasites after 1 h of incubation, a decrease in activity of the parasites incubated with the saponins was observed after 2 h, and the activity was completely abolished after 6 h of treatment (Figure 2). The findings, together with scanning electron microscopy studies (data not shown), confirm the potential of saponins to alter parasite surface via disruption of surface CPs, the key factors in their adherence to host cells.

These results were supported by gene expression studies. On treatment with *Sapindus* saponins (0.002% and 0.005%) for 3 h, there was a decline in the expression of adhesin AP65 and membrane-expressed CP TvCP2 when compared with the control *T. vaginalis* cells, while no significant effect was observed on cytosolic CP TvCP12 (Figure 3). The PCR reactions were of 25 cycles so that the results are obtained in the log phase. However, similar results were obtained when cycle number was increased from 25 to 35. No effect was also evident after treatment with *Sapindus* saponins at 0.002% concentration.

Table 1. Susceptibility of *T. vaginalis* to *Sapindus* saponins *in vitro*

Treatment and concentration	Cell numbers at different time intervals				
	3 h	6 h	12 h	24 h	48 h
Vehicle	22 500 ± 300	46 500 ± 500	165 000 ± 3500	640 000 ± 1000	2 750 000 ± 50 000
Metronidazole					
1 µM	21 500 ± 200	43 000 ± 900	158 000 ± 3000	335 000 ± 1500	1 150 000 ± 6000
3 µM	20 600 ± 600	44 250 ± 750	15 000 ± 900	216 000 ± 7000	254 000 ± 4000
6 µM	21 100 ± 500	9500 ± 250	5000 ± 300	0	0
12 µM	22 500 ± 700	6000 ± 600	1150 ± 50	0	0
Saponins					
0.0001%	22 700 ± 200	46 200 ± 250	164 500 ± 500	601 000 ± 1000	2 100 000 ± 20 000
0.0005%	22 300 ± 500	44 280 ± 280	159 250 ± 1250	506 000 ± 3000	101 500 ± 950
0.001%	21 000 ± 350	36 500 ± 500	25 300 ± 350	0	0
0.0025%	21 200 ± 100	35 325 ± 325	10 050 ± 50	0	0
0.005%	22 600 ± 400	33 200 ± 650	0	0	0

Metronidazole, the most popular drug presently available in the market, with an MIC of 6 µM³¹ was used as reference standard.

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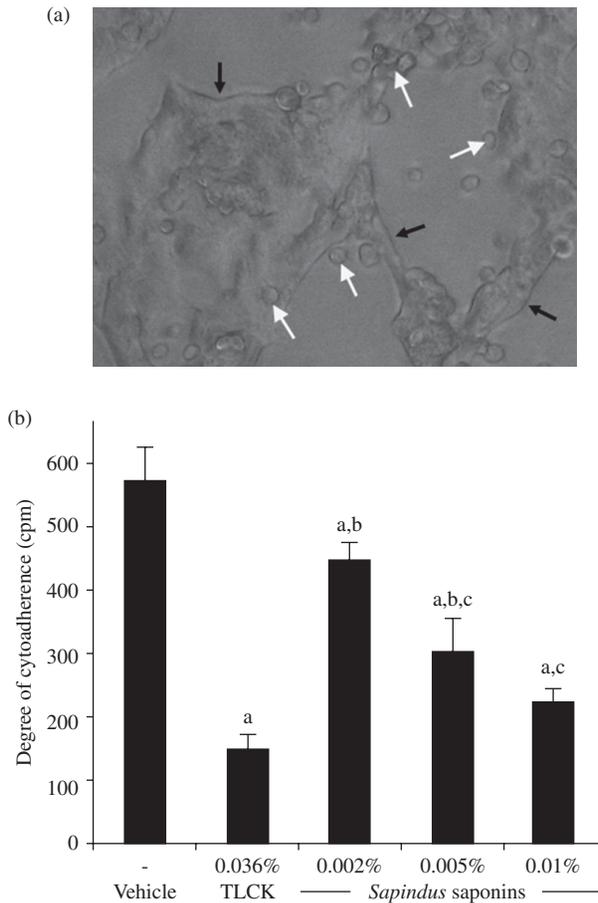


Figure 1. Cytoadherence of [³H]-thymidine-labelled *T. vaginalis* (white arrows) to HeLa cells (black arrows) *in vitro* (a). A colour version of Figure 1(a) is available as Supplementary data at *JAC* online (<http://jac.oxfordjournals.org/>). When compared with vehicle control group (0.01% DMSO), *Sapindus* saponins at MIC (0.005%) inhibited cytoadherence by ~46% (b). The inhibition (~74%) observed after treatment with TLCK (0.036%), the specific CP inhibitor, was comparable to the inhibition (~61%; $P > 0.05$) observed at 0.01% concentration (which was 5-fold lower than the spermicidal concentration) of saponins. At concentration of 0.002% of saponins, ~22% reduction in cytoadherence was observed. ^a $P < 0.01$; versus vehicle control; ^b $P < 0.01$; versus TLCK-treated group; ^c $P < 0.05$; versus preceding concentration of *Sapindus* saponins. All other relevant comparisons were statistically non-significant.

Effect on host cells

In the mitochondrial reduction potential measurement assay, there was no discernible effect on reduction potential of most of the HeLa cell population treated with the saponins at effective anti-*Trichomonas* concentration (0.005%) for 3 h (Figure 4). In comparison, HeLa cells treated with the apoptosis inducer CCCP-1 (100 μ M) produced an acute fall in their reduction potential.

However, with prolonged (24 h) exposure of HeLa cells to saponins (0.005%) in the MTT cytotoxicity assay, an ~67% cell survival rate ($P < 0.01$; versus corresponding vehicle control group; Figure 5) was observed, in comparison to only ~4% cell survival rate seen at the effective spermicidal concentration (0.002%) of the spermicide nonoxynol-9. Even at a higher concentration of 0.01%, saponins were found to be comparatively

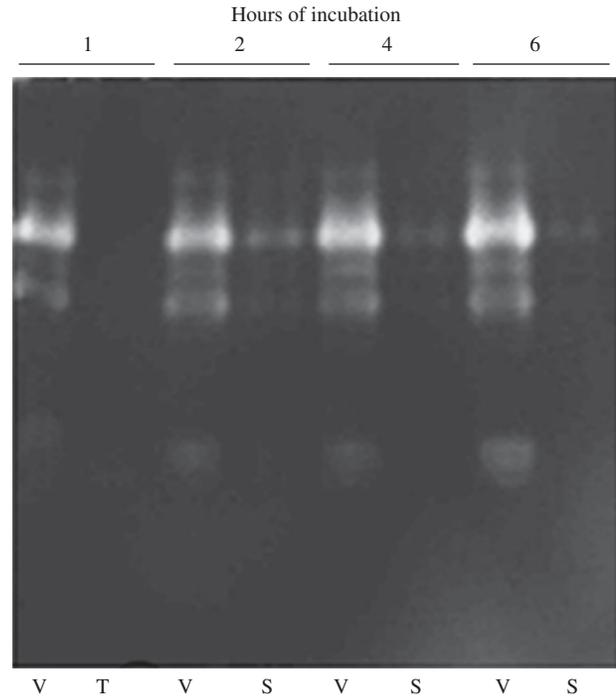


Figure 2. Substrate gel electrophoresis showing effect of *Sapindus* saponins on proteolytic activity of total CP of *T. vaginalis*. As compared with vehicle control (V; 0.01% DMSO), there was a decrease in the CP activity of the parasites with increased duration of incubation with *Sapindus* saponins (S; 0.005%), exhibiting a decrease in the activity after 2 h and its complete abolition after 6 h of treatment. TLCK (T; 0.036%), in comparison, completely abolished CP activity after 1 h of incubation.

safer (cell survival rate: ~40%; $P < 0.05$) than nonoxynol-9, while at the lower concentration of 0.002%, ~87% cell survival rate was observed after 24 h of incubation. At the 48 h time point, the survival rate of host cells in 0.005% saponins was more than 50% while no live cells could be seen in nonoxynol-9-treated samples at 0.002% concentration at the same time point (data not shown).

Inflammatory response

During interaction of RAW264.7 cells with the parasites of the vehicle control group, there was a gradual decrease in the TNF- α expression, while in the presence of saponins (0.005%), high expression level of TNF- α was observed for 4 h followed by decline at 7 h. In the case of IL-12p40, there was an up-regulated expression in RAW264.7 cells interacted with the parasites in the presence of the vehicle after 4 h of incubation. IL-12p40 expression in RAW264.7 cells interacted with parasites in the presence of saponins was delayed, and up-regulation of expression was observed only at 7 h of incubation (Figure 6). In comparison, both TNF- α and IL-12p40 expressions exhibited gradual increase with increasing duration of incubation of RAW264.7 cells in the presence of bacterial lipopolysaccharides.

Actin cytoskeleton

Cytoadherence, phagocytosis and haemolysis are important features of virulence that more or less directly depend on the

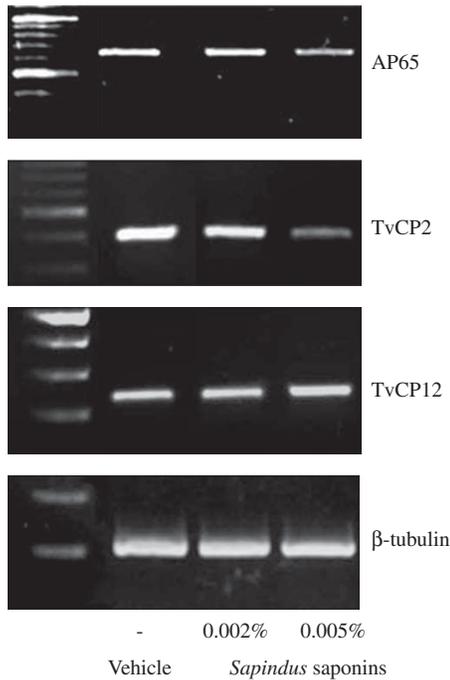


Figure 3. Differential expression of *T. vaginalis* virulence markers, adhesin AP65 and CPs TvCP2 and TvCP12 when incubated in the presence of *Sapindus* saponins for 3 h. β -Tubulin was used as the house-keeping gene. Note the decline in the expression of adhesin AP65 and membrane-expressed CP TvCP2 after treatment with *Sapindus* saponins (0.005%) when compared with vehicle control (0.01% DMSO) cells. No significant effect was observed on cytosolic CP TvCP12 at this concentration. No effect was also evident on any of the virulence markers after treatment with *Sapindus* saponins at 0.002% concentration.

cytoskeleton network of the cell of which β -actin is one of the most important components. In our studies, actin, although distributed in the entire cytoplasm, was concentrated at the periphery of the cells. In saponin-treated parasites, fluorescence at 3 and 5 h intervals was apparently reduced and concentrated mainly at the periphery, suggesting disruption of actin cytoskeleton underlying the cell membrane (Figure 7; a colour version of this figure is available as Supplementary data at JAC online (<http://jac.oxfordjournals.org/>)).

Discussion

Trichomoniasis is one of the most common sexually transmitted diseases in humans. Patients with this chronic infection are at high risk of HIV seroconversion.¹⁴ Moreover, increasing resistance to drugs such as metronidazole poses a serious problem, and new effective strategies are needed to combat this infection. Further, with an increasing population burden, there is an increased emphasis on the development of microbicides. This study demonstrates the anti-*Trichomonas* activity of *Sapindus* saponins, whose spermicidal activity has been well established.^{27,28} The *in vitro* susceptibility assay (Table 1) in the present study has shown that saponins exhibit anti-*Trichomonas* activity at a concentration 10-fold lower than its effective spermicidal concentration. This was associated with acute change in morphology from oval to a more rounded shape of

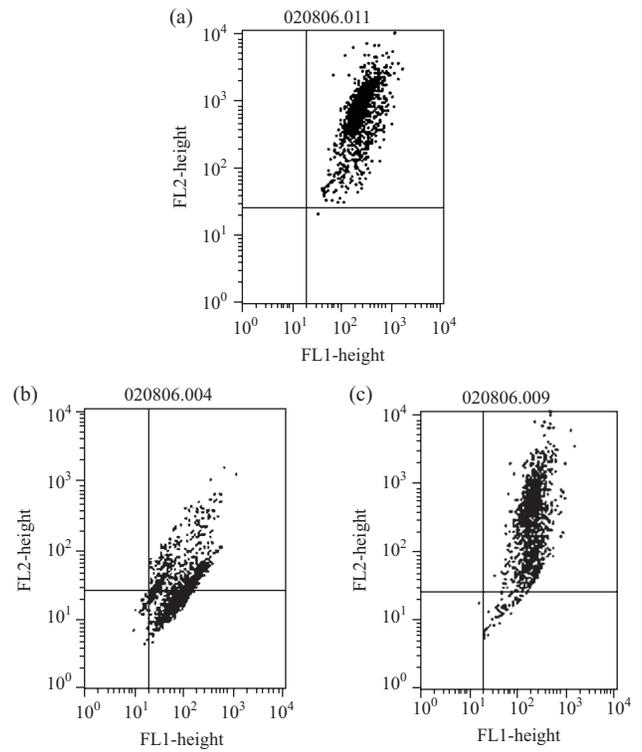


Figure 4. Dot plots showing mitochondrial reduction potential measurement study of HeLa cells using JC-1 fluorochrome after treatment with the vehicle (0.01% DMSO) (a), the known apoptosis inducer CCCP-1 (100 μ M) for 1 h (b) or *Sapindus* saponins (0.005%) for 3 h (c). The x-axis indicates intensity of first fluorescence (530 nm; emission of JC-1 monomeric form), while the y-axis indicates intensity of second fluorescence (590 nm; emission of JC-1 aggregates). Note the lack of significant effect of saponins on reduction potential of HeLa cells when compared with vehicle control cells. There was, however, an acute fall in the reduction potential of HeLa cells exposed to CCCP-1.

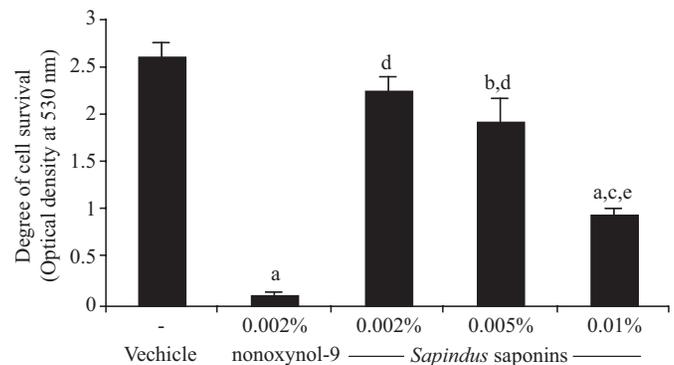


Figure 5. MTT cytotoxicity assay using HeLa cells. Cells exposed to *Sapindus* saponins (0.005%) for 24 h showed \sim 67% survival rate in comparison with only \sim 4% survival rate at effective spermicidal concentration (0.002%) of the spermicide nonoxynol-9. At a concentration of 0.01%, too, saponins were found to be comparatively safer (cell survival rate: \sim 40%) than nonoxynol-9. Vehicle control (0.01% DMSO) HeLa cells were considered as reference with 100% survival rate. ^a $P < 0.05$, ^b $P < 0.01$; versus vehicle control group; ^c $P < 0.05$, ^d $P < 0.01$; versus nonoxynol-9-treated group; ^e $P < 0.05$; versus preceding concentration of *Sapindus* saponins. All other relevant comparisons were statistically non-significant.

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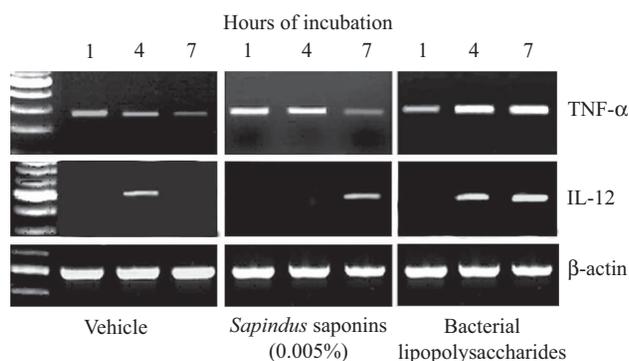


Figure 6. Effect of *Sapindus* saponins (0.005%) on expression of pro-inflammatory interleukins TNF- α and IL-12p40 in murine macrophage monocytic cell line RAW264.7 on interaction with *T. vaginalis*. When compared with cells of the vehicle control (0.01% DMSO) group, there was increased expression of TNF- α and delayed expression of IL-12p40 in the presence of the saponins. In comparison, both TNF- α and IL-12p40 expressions exhibited gradual increase with increasing duration of incubation of RAW264.7 cells in the presence of bacterial lipopolysaccharides.

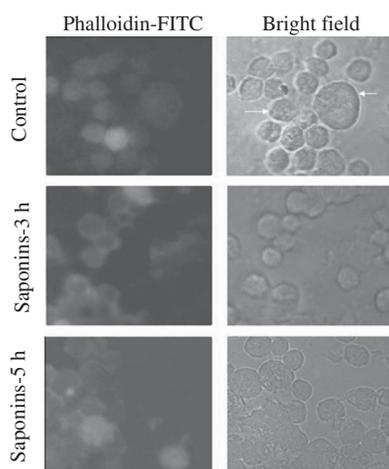


Figure 7. Effect of *Sapindus* saponins (0.005%) on actin cytoskeleton of *T. vaginalis* studied using phalloidin-FITC at 0, 3 and 5 h of incubation. Note the gradual change in the pattern of phalloidin staining with an increase in the duration of incubation of parasites with the saponins indicating disruption of actin cytoskeleton underlying the cell membrane after 3 h of incubation. A colour version of this figure is available as Supplementary data at JAC online (<http://jac.oxfordjournals.org/>).

the parasites before cell death, as has also been reported in the case of pseudocysts that appear under unfavourable environmental conditions when the flagella are internalized and a true cell wall is not formed.³³ In axenic cultures, this protozoan appears oval- or pear-shaped,¹³ but takes a more amoeboid appearance when attached to vaginal epithelial cell surface.¹⁰ Pertinently, at this concentration, saponins are neither cytotoxic to the host cells nor do they alter vaginal microflora.³⁴

Trichomonad cytopathogenicity is a multifactorial process involving events such as cytoadherence and immune evasion. Cytoadherence is a key property for colonization and infection by *T. vaginalis* and is mediated principally by two groups of molecules named adhesins¹¹ and CPs,¹³ which are expressed on

the parasite surface. Trichomonad CPs have been related to nutrient acquisition, immune evasion and virulence properties such as cytoadherence, haemolysis and cytotoxicity.^{5,14,35–37} Besides, iron is an important nutrient for *T. vaginalis*, which it is unable to synthesize on its own and must acquire it via lysis of erythrocytes.³⁸ Hence, haemolytic activity of the parasites can also be correlated with their virulence. Results of the present study also show that saponins inhibit adherence of *T. vaginalis* to the host cell surface, a prerequisite for the initiation of infection. Saponins are detergents, and there is a possibility that detergent action may affect the membrane bilipid layer and lead to a decrease in the cytoadherence. However, this does not seem to be the case as parasites were treated with saponins for a shorter time span, i.e. 1–3 h, and their morphology and motility were the same as that of the untreated parasites. Saponins also inhibit proteolytic activity of parasite's CPs that are important for adherence, nutrition acquisition and virulence of the parasites. AP65 is an important adhesin targeted to both surface membrane and hydrogenosome and is centrally involved in cytoadherence.^{29,39} The putative TvCP12 protein has no signal peptide,⁴⁰ suggesting that this is a cytoplasmic protein, while TvCP2 has been thought to have membrane location.³⁷ Hence, decreased expression of AP65 and TvCP2 observed in the present study supports the inhibition of adhesins and membrane-expressed CPs at the genetic level while there is relatively no effect on cytosolic TvCP12.

Another strategy followed by *T. vaginalis* for colonization and persistent infection is evasion of the host immune response. The mucosal immune system is the first stage of defence against pathogenic organisms in the female reproductive tract. It involves both innate and adaptive immune responses including humoral and cell-mediated immunity, with the innate immune system playing a particularly crucial role in the resistance to a variety of protozoa during early stages of infection.⁴¹ Studies have shown that *T. vaginalis* induces a rapid activation of NF- κ B in RAW264.7 macrophages during the early stage of adhesion. However, this activation is not maintained but leads to inhibition of the production of pro-inflammatory cytokines.⁴¹ Furthermore, *T. vaginalis* infection induces a state of non-responsiveness to subsequent stimulation with bacterial lipopolysaccharides. These results suggest that *T. vaginalis* induces an inhibitory mechanism that prevents or delays the immune response of the host cells. Pertinently, macrophages are important components of the innate immune system and are capable of producing TNF- α and IL-12p40.⁴¹ According to Chang *et al.*,⁴¹ pro-inflammatory cytokines in response to the *T. vaginalis* show a definite pattern. It was seen that *T. vaginalis* shows an immune evasive response. These investigators, using quantitative real-time PCR, revealed that the expression of TNF- α and IL-12 mRNA in *T. vaginalis* adhesive cells was rapidly suppressed in comparison to lipopolysaccharides stimulation. In the present study, aimed to determine the effect of saponins on *Trichomonas*-induced immunity using RAW264.7 macrophage cells infected with *T. vaginalis* (1:10), saponins prevented suppression of pro-inflammatory cytokines induced by *T. vaginalis*, suggesting one of the possible mechanisms of action of saponins against *Trichomonas*.

Previous studies have also shown that treatment of *T. vaginalis* with pro-apoptotic drugs and metronidazole leads to a form of non-necrotic cell death with some features resembling apoptosis.⁴² The use of caspase inhibitors that abolish apoptotic process

in *T. vaginalis* strongly suggests the presence of caspase-like proteinases in this microorganism.⁴² The findings of our study using Hoechst staining and DNA electrophoresis, however, did not reveal any difference between untreated and saponin-treated parasites. In fact, fluorescing nuclei were evident in saponin-treated parasites even after 3 h of treatment (data not shown). The flagellate form of *T. vaginalis* is known to transform to an amoeboid form upon adherence to coverslips. Although they grow, their nuclei divide without undergoing cytokinesis, yielding giant cells,^{43,44} and the presence of a monolayer of F-actin has been demonstrated in *T. vaginalis* by fluorescence microscopy using phalloidin and an anti-actin monoclonal antibody that labelled cytoplasm of both the flagellate and amoeboid forms. In the present study, treatment of parasites with the saponins resulted in decreased fluorescence in cytoplasm when compared with untreated control parasites. Findings indicate that *Sapindus* saponins exert their anti-*Trichomonas* activity via disruption of cytoskeletal network rather than via DNA damage, as caused by metronidazole.

In conclusion, the present study demonstrates that saponins from *Sapindus mukorossi* exhibit anti-*Trichomonas* activity at 10-fold lower concentration than the effective spermicidal concentration against human spermatozoa and suggests potential of saponins for development as spermicidal microbicide for human use. Further studies are, however, required to evaluate its microbicidal activity against other sexually transmitted infections.

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Transparency declarations

None to declare.

Supplementary data

Colour versions of Figures 1 and 7 are available as Supplementary data at JAC online (<http://jac.oxfordjournals.org/>).

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