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 florescence-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...
adherence of *T. vaginalis* to vaginal epithelial cells has been suggested.13 Pre-treating trichomonads with *N*-tosyl-1-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 lysosomal15,16 and have been implicated as probable lytic factors in the haemolysis of erythrocytes.17–19

The classical 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 result 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 mukurozisapinon 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* Gaeth (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 sapogenins linked to a particular sequence of sugar moieties.29 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-mediatated adherence of *Trichomonas* to the host cells.
TYI-S33 medium to remove unincorporated $[^3]H$-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% 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, $1 \times 10^6 [^3]H$-thymidine-labelled trichomonad trophozoites treated with the saponins or the vehicle (0.01% DMSO) were allowed to interact with $2 \times 10^5$ 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 $[^3]H$-thymidine was measured on an LS Analyzer 6500 (Beckman Instruments Inc., USA).

CP activity

Substrate gel electrophoresis$^{32}$ for the estimation of the activity of CPs of Trichomonas was used with slight modifications. Briefly, $1 \times 10^6$ 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 MgCl$_2$, 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. $5 \times 10^5$ HeLa cells grown in DMEM supplemented with 10% FCS at 37°C under 5% CO$_2$ 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% CO$_2$. The medium was then aspirated and formazane 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 μg/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 GCT GGA TG-3' (antisense), 450 bp; for TvCP12, 5'-GAT TTC AAC CCT CTT CCG GCA TT-3' (sense), 5'-GTT GAC TGT TGG CCC GCT GGA AA-3' (antisense), 270 bp; for β-tubulin, 5'-CAT TGA TAA CCA AGC TCT TTA CGA T-3' (sense), 5'-GCA TGT TGT GCC GCA GAT AAC CAT-3' (antisense), 300 bp; for TNF-α, 5'-ATG ACA AGA ACC ATG TTC ATC-3' (sense), 5'-TAC AGG CTG AGC CTT CGA ATT-3' (antisense), 276 bp; for IL-12p40, 5'-ATG TCC TGG TTG GCC ATC GTT TTG-3' (sense), 5'-GCC CTT TGG TCG AGT GTG ACC TTC-3' (antisense), 527 bp and for β-actin, 5'-TGT GAT GGG AAT GGG TCA-3' (sense), 5'-TTT GAT GTC ACG CAC GAT TCC C-3' (antisense), 514 bp. Amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA).

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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,30 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 TpCP2 when compared with the control *T. vaginalis* cells, while no significant effect was observed on cytosolic CP TpCP12 (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**

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

Metronidazole, the most popular drug presently available in the market, with an MIC of 6 μM was used as reference standard.
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-\textit{Trichomonas} concentration (0.005%) for 3 h (Figure 4). In comparison, HeLa cells treated with the apoptosis inducer CCCP-1 (100 μM) 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 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...
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. 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 microbicidal contraceptives. This study demonstrates the anti-Trichomonas activity of Sapindus saponins, whose spermicidal activity has been well established. 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 the cell. The effect was also evident on any of the virulence markers after treatment with Sapindus saponins at 0.002% concentration.

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.

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 μM) 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 ~67% survival rate in comparison with only ~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: ~40%) than nonoxynol-9. Vehicle control (0.01% DMSO) HeLa cells were considered as reference with 100% survival rate. Values are presented as mean percentage of survival ± SEM. a,b,c,d,eP < 0.05, a,b,cP < 0.01; versus vehicle control group; aP < 0.05, a,bP < 0.01; versus nonoxynol-9-treated group; b,c,dP < 0.05; versus preceding concentration of Sapindus saponins. All other relevant comparisons were statistically non-significant.
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. In axenic cultures, this protozoan appears oval- or pear-shaped, but takes a more amoeboid appearance when attached to vaginal epithelial cell surface. Pertinently, at this concentration, saponins are neither cytotoxic to the host cells nor do they alter vaginal microflora.

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 adhesins and CPs, 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. 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. 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. The putative TvCP12 protein has no signal peptide, suggesting that this is a cytoplasmic protein, while TvCP2 has been thought to have membrane location. 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. Studies have shown that *T. vaginalis* induces a rapid activation of NF-κB 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. 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. According to Chang et al., pro-inflammatory cytokines in response to *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 lipopolysaccharide 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. The use of caspase inhibitors that abolish apoptotic process.

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-12 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 of 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. In axenic cultures, this protozoan appears oval- or pear-shaped, but takes a more amoeboid appearance when attached to vaginal epithelial cell surface. Pertinently, at this concentration, saponins are neither cytotoxic to the host cells nor do they alter vaginal microflora.

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 adhesins and CPs, 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. 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. 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. The putative TvCP12 protein has no signal peptide, suggesting that this is a cytoplasmic protein, while TvCP2 has been thought to have membrane location. 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.

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in T. vaginalis strongly suggests the presence of caspase-like proteases in this microorganism. 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, 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 spermidial 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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