Sperm motility inhibiting activity of a phytosterol from Alstonia macrophylla Wall ex A. DC. leaf extract: A tribal medicine

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The role of methanolic extract and n-butanol fraction of A. macrophylla leaves was investigated on the forward motility of goat spermatozoa. The methanol extract (600 µg/ml) and one n-butanol fraction (Fraction A; 100 µg/ml) showed marked inhibition of sperm forward motility, tested by microscopic and spectrophotometric methods. Approximately, 50-60% of the spermatozoa lost their motility when treated with 600 µg/ml of methanol extract or 100 µg/ml of Fraction A. The Fraction A at 400 µg/ml concentration showed complete inhibition of sperm forward motility at 0 min. The inhibitory activity increased with the increasing concentrations of the fraction. The motility inhibitory activity of the Fraction A was stable to heat treatment at 100°C for 2 min. The compound showed high inhibitory effect in the pH range 6.7-7.6. Fraction A also showed high efficacy for inhibiting human sperm motility, assessed by the microscopic method. The phytochemical analysis of methanolic extract of A. macrophylla leaves revealed the presence of sterols, triterpene, flavonoid, alkaloid, tannin and reducing sugar, while the Fraction A contains β-sitosterol, a common phytosterol. The results demonstrate that Fraction A (β-sitosterol) is a potent inhibitor of sperm motility and thus it has the potential to serve as a vaginal contraceptive.

Keywords: Alstonia macrophylla, β-sitosterol, Motility inhibition, Spermatozoa

Spermatozoa are highly specialized cells. Mammalian spermatozoa have flagella, which keep the sperm mobile and help in transport of sperm from male reproductive tract to the female tract during copulation. Fundamental understanding of the mechanism and function of flagellum is derived primarily from examination of protozoan cilia and the tail of echinoderm spermatozoa1,2. Flagellar movements have been well studied in different species3. Sperm hyperactivated motility has potential importance in fertilization3,4, because sperm motility, binding of spermatozoa to the zona pellucida, and induction of acrosome reaction are the prerequisites for successful oocyte fertilization.

Nature has gifted mankind with abundant natural remedies in the form of herbs, shrubs, trees and mineral elements. The use of plants as medicaments is as old as the mankind. The crude extracts of Alstonia macrophylla leaf and stem bark are used as medicaments by the tribal populations of Bay Islands for stomachache, skin diseases, urinary and gastrointestinal ailments since time immemorial8-10. However, the recent studies on A. macrophylla showed that the alcoholic extract of leaves could inhibit several bacteria, and a few dermatophytic fungi that can cause stomachache, skin diseases and urinary problems in man11. The most interesting observation is that the methanolic leaf extract of A. macrophylla at 8-16 µg ml−1 concentration can inhibit12 the swarming of all the tested Proteus species, having peritrichous flagella. These findings indicated that the active compound may be used as an anti-swarming agent for microbial cultures, and this also prompted us to investigate its effect on the motility of mammalian spermatozoa. A number of plant extracts have antifertility effects13 and Azadirachta indica oil, commonly known as neem oil, is reported to be a vaginal contraceptive14, while its leaf extract can cause changes in
morphology (in head region), motility and functions of rat spermatozoa. The aim of the present study is to know whether the extract or any fractions of *A. macrophylla* leaf can inhibit the forward motility of mature spermatozoa. For this, the effect of the extract has been studied on forward motility of (goat) spermatozoa in the presence of epididymal plasma (EP) to rule out the possibility of “cell-sticking” artefact in motility assay.

**Materials and Methods**

*Extraction and fractionation*— The leaves of *Alstonia macrophylla* Wall ex A. DC (Apocynaceae) were collected from the rain forests of Middle and South Andamans, India, during April, June and October 1999 as well as in May, July and November 2000. The voucher specimens were authenticated and deposited at the Herbarium Repository of the Botanical Survey of India, Andaman & Nicobar Circle, Port Blair, India. Coarsely powdered dry leaves (1 kg) were successively extracted with 95% cold methanol as solvent for 72 hr at room temperature. The whole extract was collected in a 5 litre conical flask, filtered and the solvent was evaporated to dryness under reduced pressure in an Eyela Rotary Evaporator (Japan) at 40°-45°C. The w/w yield of the prepared extract was 8.9 ± 0.21%. The concentrated extracts were then partitioned between *n*-butanol and *H*₂*O*, while the aqueous portion was lyophilized to dryness (~30 g). The phytochemical tests indicated the presence of tannins, triterpenoids, flavonoids, sterols, alcohols and sugars. Simultaneously, the solvent of *n*-butanol part was evaporated under reduced pressure in a Rotary Evaporator at ~45°C. The *n*-butanol fraction, weighing ~25 g, was then purified on silica gel (60-120 mesh, SRL) by Column Chromatography, and eluted with petroleum ether (PE) : CHCl₃ mixture (at different ratios), CHCl₃ : MeOH mixture (at different ratios) and MeOH. All the eluted fractions were then monitored by Thin Layer Chromatography (TLC). Three major compounds A, B and C were isolated along with a mixture of minor compounds (detected in TLC only). The isolated major compounds were then purified by repeated silica gel column chromatography and were eluted by PE : CHCl₃ (1:1) mixture and CHCl₃ : MeOH (95:5) mixture. The spectral data (IR, Mass and NMR) of A, B and C were identical with β-sitosterol, ursolic acid and β-sitosterol glucoside, respectively. The identification of these compounds was also done by Co-TLC and spiperimposable IR with authentic samples. Melting points were checked by mixed samples i.e., authentic and isolated compounds, for all these compounds.

*Reagents*— Penicillin G and potassium phosphate (dibasic, anhydrous) were obtained from Sigma Chemical Co., USA. Other chemicals used were of reagent grade. Fresh epididymes of adult goat were obtained from the local slaughter houses. Human semen ejaculates were obtained from the healthy male volunteer recruited for the purpose.

*Isolation of spermatozoa and epididymal plasma*— Goat epididymal spermatozoa were isolated within 2-4 hr of slaughter. The cauda part of the epididymis was minced and suspended in a modified Ringer’s solution (Ringer phosphate solution (RPS) medium containing: 119 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 10 mM glucose, 16.3 mM potassium phosphate, and 50 U penicillin ml⁻¹, pH 6.9). Highly motile spermatozoa were obtained, and numbers of spermatozoa in the sample were estimated with a haemocytometer. Freshly extracted sperm preparations contained 10-20 x 10⁶ cells ml⁻¹. Goat epididymal plasma was prepared by centrifugation of freshly extracted sperm suspension at 800 g for 10 min. The resultant supernatant (slightly turbid) was spun again at 14000 x g for 10 min to obtain cell free EP. The concentration of EP in the assays was expressed as its protein content, estimated according to Lowry et al. with bovine serum albumin as standard.

*Assay of forward motility inhibiting activity*—

*Microscopic assay*— Action of the methanol extract and Fraction A on forward motility (FM) of goat and human sperm was estimated by conventional microscopic method using haemocytometer as a counting chamber. To rule out the possibility of sperm adhesion to glass, motility assays were carried out in presence of EP (1 mg protein ml⁻¹) which causes nearly 100% inhibition of sperm adhesion to glass. Spermatozoa (1 x 10⁶ cells) were incubated with EP (1.2 mg protein ml⁻¹) in presence or absence of methanol extract and Fraction A at room temperature (32° ± 1°C) for 2 min, in a total volume of 0.5 ml of RPS. A portion of cell suspension was then placed in the haemocytometer. Forward motile cells and the total number of cells were counted under a phase contrast microscope at 400× magnification. The percentage of forward motile cells was then calculated. A unit of activity of the motility inhibition was defined as the concentration of the
fraction, which inhibited FM in 10% of the cells under the standard assay conditions. The data were expressed as mean ± SE of at least 3 experiments.

Spectrophotometric assay
Forward motility of spermatozoa was also estimated by a quantitative method\(^2\),\(^3\). The microscopic method of motility assay described above takes into consideration the number of cells with forward progression but not their velocity, whereas the spectrophotometric method was based not only on the motile cell number but also on their velocity. The method consisted of layering 50 µl of freshly extracted cauda epididymal spermatozoa (2.6 \times 10^6 cells ml\(^{-1}\)) suspended in RPS medium containing 2% Ficoll-400 at the bottom of a standard optical cuvette (3 ml capacity) containing 1.3 ml of RPS medium which was sufficient to cover the entire width of the light beam. Vigorously motile spermatozoa that moved upward and entered in the light beam were registered continuously as an increase of absorbance at 545 nm with a spectrophotometer equipped with the recorder. After reaching the maximum absorbance (\(A_{mg}\)), the content of the cuvette was mixed and the absorbance for all the cells was noted (\(A_T\)). The percentage of cells that showed vigorous forward motility was calculated as \(\frac{A_{mg}}{A_T} \times 100\).

Results
Cold methanol extract of the dried, powdered leaves of \(A.\) \(m\)acrophyl\(a\) yielded, upon removal of the solvent, a dark viscous brown mass. At 8-16 µg ml\(^{-1}\) concentration, it demonstrated good in vitro antitwarming activity against 32 strains of \(P.\) \(m\)irabil\(is\) and 18 strains of \(P.\) \(v\)ulgar\(is\).

Different concentrations of the methanol extract and the Fraction A were tested on FM of goat and human spermatozoa. Freshly extracted goat cauda-sperm preparations showed 30-50% FM in presence of EP (1.2 mg protein ml\(^{-1}\)). Epididymal plasma contains adequate amount of antisticking factors, which helps to avoid adhesion of sperm cells to the glass surface of haemocytometer. Fraction A at the concentration of 100 µg ml\(^{-1}\) caused nearly 60% inhibition (\(p<0.01\)) of goat sperm flagellar motility, while the motility inhibiting activity of methanol extract at 660 µg ml\(^{-1}\) concentration was 60-70%. The motility inhibiting activity of Fraction A increased linearly up to 8 units at 200 µg ml\(^{-1}\) (0.2mg ml\(^{-1}\)) concentration. The sperm FM was completely inhibited at the concentration of 400 µg ml\(^{-1}\) (0.4mg ml\(^{-1}\)) of Fraction A, while the concentration required for complete motility inhibition by crude methanol extract was 1000 µg ml\(^{-1}\) (Fig. 1). A dose dependent curve showed that the inhibitory action of Fraction A increases with the increasing concentration (Fig. 2). The microscopic method of motility assay gave a subjective assessment of sperm motility. The results of the FM inhibiting activity of the crude extract and its fractions by spectrophotometric method revealed that the Fraction A

![Fig. 1](image1.png)

**Fig. 1**—Effect of varying concentrations of methanol extract of \(A.\) \(m\)acrophyl\(a\) leaf extract on the forward motility of goat cauda spermatozoa under standard assay condition. [\(\bullet\) forward motility (%); \(\blacktriangle\) MI activity unit. The values are the mean ± SE of 3 experiments.]

![Fig. 2](image2.png)

**Fig. 2**—Effect of varying concentrations of Fraction A on goat cauda sperm forward motility under the standard assay conditions. [\(\bullet\) forward motility (%); \(\blacktriangle\) MI Activity unit. The values are mean ± SE of the 3 experiments (\(p<0.01\)).]
strongly inhibited the FM of goat spermatozoa (Fig. 3). The control cells showed 40% vigorous FM, whereas the addition of Fraction A at 100 and 200 µg ml⁻¹ concentrations reduced sperm motility to 30 and 18%, respectively.

The sperm FM was totally inhibited at 0 min at 0.4 mg ml⁻¹ concentration of Fraction A. It showed nearly 100% inhibition of sperm FM at 200 µg ml⁻¹ (0.2 mg ml⁻¹) concentration after 15 min of incubation at room temperature (Fig. 4). When the Fraction A was tested on human sperm, it caused 90% inhibition of FM at 100 µg ml⁻¹ concentration (Table 1). The sperm motility inhibitory activity of the Fraction A was found to be stable to heat treatment at 80 and 100°C for 2 min (Table 2). The inhibitory action of the Fraction A (200 µg ml⁻¹) was nearly same (approx. 75%) at the pH range 6.7-7.6 (data not shown) thereby showing that the fraction is potentially active at this pH range. The concentration dependent inhibitory action of Fraction A on FM of human spermatozoa was more pronounced than the goat cauda spermatozoa.

Discussion

The results of the present study show that the methanol extract of A. macrophylla leaf and its fraction effectively immobilize forward motility of mammalian spermatozoa. The Fraction A of methanol extract possesses a highly effective compound that inhibits the FM of goat cauda spermatozoa and human spermatozoa. At 400 µg ml⁻¹ (0.4 mg ml⁻¹) concentration Fraction A inhibits 100% sperm motility at “0” min, while a minimum concentration of 100 µg ml⁻¹ of Fraction A causes immobilization of total sperm cells in 30 min at room temperature. These observations indicated that the Fraction A is a potent inhibitor of sperm FM.

The leaf extract of A. macrophylla contains alkaloids like picrinine, picalstonine, β-benzoyl vincamajine and quebrachidine. The recent phytochemical study of n-butanol fraction of methanol extract, however, showed the presence of β-sitosterol (Fraction A), ursolic acid (Fraction B) and β-sitosterol glucoside (Fraction C) as major compounds.

Table 1—Effect of different concentrations of Fraction A of A. macrophylla leaf extract on forward motility of human sperm [values are mean ± SE]

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Forward motility (%)</th>
<th>Inhibition of motility (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>35 ± 1.70</td>
<td>-</td>
</tr>
<tr>
<td>+ Fraction A (100µg/ml)</td>
<td>7 ± 0.88</td>
<td>80 ± 2.08</td>
</tr>
<tr>
<td>+ Fraction A (200µg/ml)</td>
<td>3 ± 1.2</td>
<td>92 ± 3.6</td>
</tr>
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Table 2—Effect of heat treatment on forward motility inhibiting activity of fraction A at 400 µg/ml of A. macrophylla leaf extract on goat sperm

<table>
<thead>
<tr>
<th>Heat (°C)</th>
<th>Forward motility (%)</th>
<th>Motility inhibition (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>54</td>
<td>Nil</td>
</tr>
<tr>
<td>28 (Room Temp.)</td>
<td>4</td>
<td>9.2</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>9.6</td>
</tr>
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</table>

*Control- System without Fraction A
along with a mixture of minor compounds only detected in TLC. The plants have the ability to synthesize a number of sterols of which the most common are campesterol, sitosterol and stigmasterol. These sterols contain an extra alkyl group at C-24 position in the side chain. In all higher plants these sterols occur free, as esters and β-D glucosides as primary biosynthetic product. In cell membrane, the bulkier sitosterol and stigmasterol have conformational and permeability implications. In human, sitosterol (24α-ethylcholesterol) acts as a plasminogen activator and promotes the formation of essential polyunsaturated fatty acids from linoleic acid which are required for prostaglandin and leukotriene synthesis, and are important for cell mediated immune functions. Sitosterol itself has several biological effects. It has been shown to be effective in inhibiting HT 29 human colon cancer cell growth and epithelial cell proliferation and chemically induced colon tumours in rats and mammary lesions in organ culture; further, it is also shown as an antimutagenic agent. Sitosterol and its glucoside with a synergistic enhancement when both are given together have a proliferating effect on T-cell production in vitro at remarkably low level of 10 and 1 pg l⁻¹ (24 and 1.7 fM) respectively. An enhanced T-cell proliferative response was observed after 4 weeks of daily oral supplementation with 60 mg sitosterol and 0.6 mg of its glucoside in human volunteers with a normal diet. This strongly suggests that plant sterols, particularly sitosterol and its glucoside, have beneficial effect on immune system at surprisingly low concentrations. It is also known that sitosterol has anti-inflammatory, antiulcer, anti-diabetic, and anticancer activities.

The β-sitosterol present in Fraction A of the crude methanolic extract of A. macrophylla leaf appears to be a potential agent for developing tropical contraceptive. The saponins and terpenes, widely available in plants, are reported to have spermicidal activity and the water soluble saponin fraction isolated from Acacia auriculiformis is found to have significant sperm immobilizing activity, though their use as vaginal contraceptive is yet to be developed. Alstonia macrophylla leaf extract has antibacterial and antifungal properties. Thus, this plant, besides being spermicidal, may have protective role against some sexually transmitted infections. The exact mechanism by which the extract arrested FM of mammalian spermatozoa remains poorly understood and this requires further investigation with purified compound.

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References


