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ANTIMOTILITY EFFECTS OF *PIPER BETELE* (L.) LEAF EXTRACT ON WASHED HUMAN SPERMATOZOA

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Abstract : Polyvinylpyrrolidone (10,000 MW) co–precipitate of dichloromethane: methanol (1:1) extract of *Piper betele* (L.) (Piperaceae) leaves were investigated (N=12) for antimotility effects on washed human spermatozoa (up to 60 min. of incubation) using three parameters (percentage motility, average forward velocity and motility index). The concenrtrations tested were 0.25, 0.5, 1.25, 2.5 and 5.0 mg/ml. The extract impaired these motility parameters in a dose related manner. The concentrations producing 50% inhibition were : percentage motility 0.47 ± 0.11 mg/ml, average forward velocity 0.37 ± 0.07 mg/ml and motility index 0.31 ± 0.02 mg/ml (mean ± s.e.m.). Futhermore, the antimotility effects were irreversible and were not accompanied either with changes in the fluidity of the plasma membrane (hypo–osmotic swelling test) or in the viability (nigrosin–eosin stain) of spermatozoa. The results suggest that potent antimotility agent/s may be isolated from *Piper betele* (L.) leaves.

1. Introduction

The search for new and effective forms of vaginal contraceptives continues. Several synthetic[12] and natural products[3,11,14,16] have been tested as potential vaginal contraceptive agents. In this context, we have also initiated a programme to develop vaginal contraceptive agents from locally available plant sources. This study reports the inhibitory effects of the crude extract of *Piper betele* (L.) (Piperaceae) on washed human spermatozoa.

2. Experimental

2.1 Preparation of Leaf Extract

500g of mature fresh leaves of *Piper betele* were plucked in early June, 1988, from a plantation situated in the wet zone of Sri Lanka. These were cut into small strips (about 5 mm width) and macerated using a domestic blender in one litre of distilled water for 15 min. The supernatant was then filtered off using a muslin cloth, and partitioned with an equal volume of dichloromethane:methanol (1:1) solvent system for 48h. The organic layer was decanted and concentrated under negative pressure at 30 ± 1°C to obtain the crude extract (yield ; 0.423 mg/g). Polyvinylpyrrolidone (PVP) (MW–10,000) co-precipitate of the extract was prepared by concentrating a methanolic solution of the crude extract (1.0 mg/ml) and PVP in the ratio of 1:4 (W/W). The solvents were then removed *in vacuo* at 30 ± 1°C utilizing a rotary evaporator.

2.2 Semen Samples

Semen samples were obtained from normal healthy volunteers by masturbation into plastic containers after at least 72 h. of sexual abstinence. The specimens were allowed to liquify at room temperature (30 ± 1°C) for 15 – 25 min. and then semen analysis was undertaken under phase contrast microscopy according to the guidelines established by the World Health Organization.[17] Only donors with normal semen profiles (⩾40 x 10^6 spermatozoa/ml, ⩾40% motility, ⩾60% normal morphology) were used (N=12). The spermatozoa were isolated from seminal plasma by three cycles of centrifugation (500g for 5 min.) with 9 ml volumes of medium Biggers, Whitten and Whittingham[1] (BWW) and were finally resuspended in BWW at a density of 20 x 10^6 spermatozoa/ml for the evaluation of the effects of the *Piper betele* leaf extract on spermatozoa.

2.3 Evaluation of Antimotility Activity

The PVP co-precipitate of the extract was dissolved in BWW medium (test solution; stock concentration 20 mg/ml) and its pH was determined using a pH meter (Chemtrix type 40). 500 µl of the washed spermatozoa in BWW was placed in Falcon tubes and an equal volume of PVP dissolved in BWW (final concentration 20 mg/ml) (vehicle) or the test solution was added (final concentration of test solutions were 0.25,0.5,1.25,2.5 and 5.0 mg/ml), mixed well and the time was recorded (0 min.). 10 µl of this suspension was transferred on to a clean glass slide (31 ± 1°C) and covered with a coverglass (22 x 22 mm). Immediately and at 5, 10, 15, 30, and 60 min. the percentage motile spermatozoa[17] and the average forward velocity[4] were determined at each concentration under phase contrast microscopy using a square grid and a crossed calibrated micrometer, respectively. The motility index was then computed as the product of the above two motility parameters.[10] The concentration of the extract that decreased to 50% of control (EC_{50}), the percentage motile spermatozoa, the average forward velocity and the motility index was calculated from log. concentration response curves. The spermatozoa incubated with 5.0 mg/ml test solution for 5 min. were washed thrice with BWW medium and the above mentioned three spermatozoal movement characteristics were reinvestigated. The samples with 2.5 mg/ml test solution and the vehicle were subjected to the hypo–osmotic swelling test[8] and to nigrosin–eosin stain technique[17] at 0 and 60 min. respectively. The results are represented as mean ± s.e.m. Statistical analysis were made using either Duncan's multiple range test of Mann–Whitney U–test.

3. Results

The pH of the stock solution of the extract was 7.0. The extract neither caused flocculation of spermatozoal suspension nor agglutination of spermatozoa. The results of the motility experiments are summarized in figures 1,2 and 3. A significant inhibition (Duncan's multiple range test, $p < 0.05$)

of forward velocity and motility index was evident at all concentrations and incubation times. This was also the case for the percentage motility, except at the lowest dose (0.25 mg/ml), where a significant reduction ($p < 0.05$) was seen only following 5 min. of incubation. In addition, at the highest dose (5.0 mg/ml) the motility parameters were totally inhibited in all the samples. Repeated washing of samples treated with the highest dose for 5 min. failed to revive any of the three spermatozoal movement characteristics. The EC_{50} values for the extract were : impairment of percentage motility 0.47 ± 0.11 mg/ml, average forward velocity 0.37 ± 0.07 mg/ml and motility index 0.31 ± 0.02 mg/ml.

In the hypo–osmotic swelling test, the percentage, swollen spermatozoa in the control were 72.4 ± 6.5 (0 min) and 59.4 ± 5.9 (60 min.). The corresponding values with 2.5 mg/ml extract were 65.2 ± 6.9 and 66.0 ± 7.9 respectively. This effect was not significant (Mann–Whitney U–test); 2.5 mg/ml extract also did not significantly (Mann–Whitney U–test) reduce the viability of spermatozoa as revealed from the nigrosin–eosin stain technique; the percentage of live spermatozoa at 0 and 60 min. in control were 76.0 ± 6.29 and 76.3 ± 5.4 respectively and with extract were 75.8 ± 6.3 and 68.2 ± 6.6 respectively.

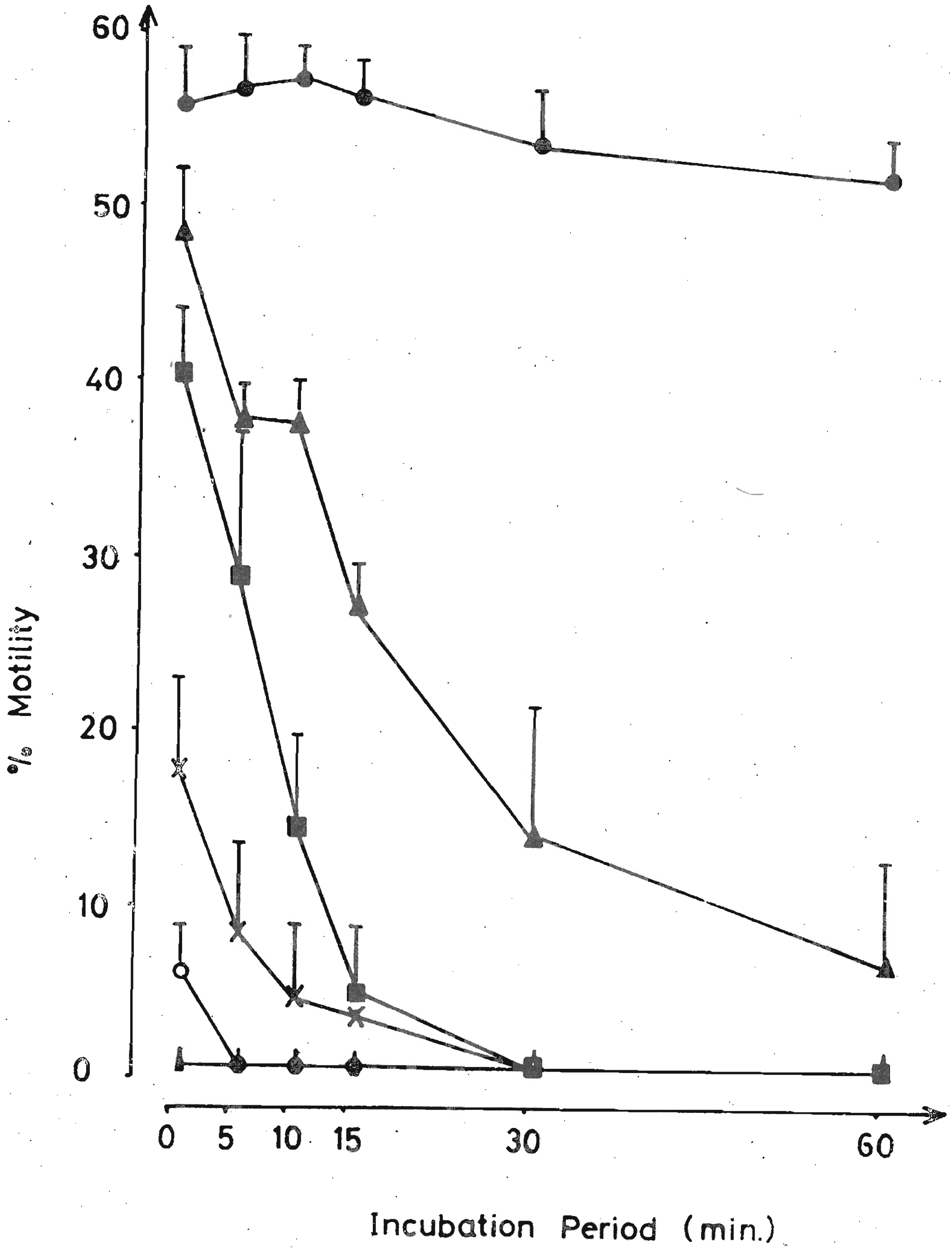

Figure 1 : Inhibition of percentage motility of washed human spermatozoa by *Piper betele* leaf extract.

● = control ; ▲ = 0.25 mg ml^{-1} ■ = 0.5 mg ml^{-1} ;
x = 1.25 mg ml^{-1} ; ○ = 2.5 mg ml^{-1} ; ▲ = 5.0 mg ml^{-1}.

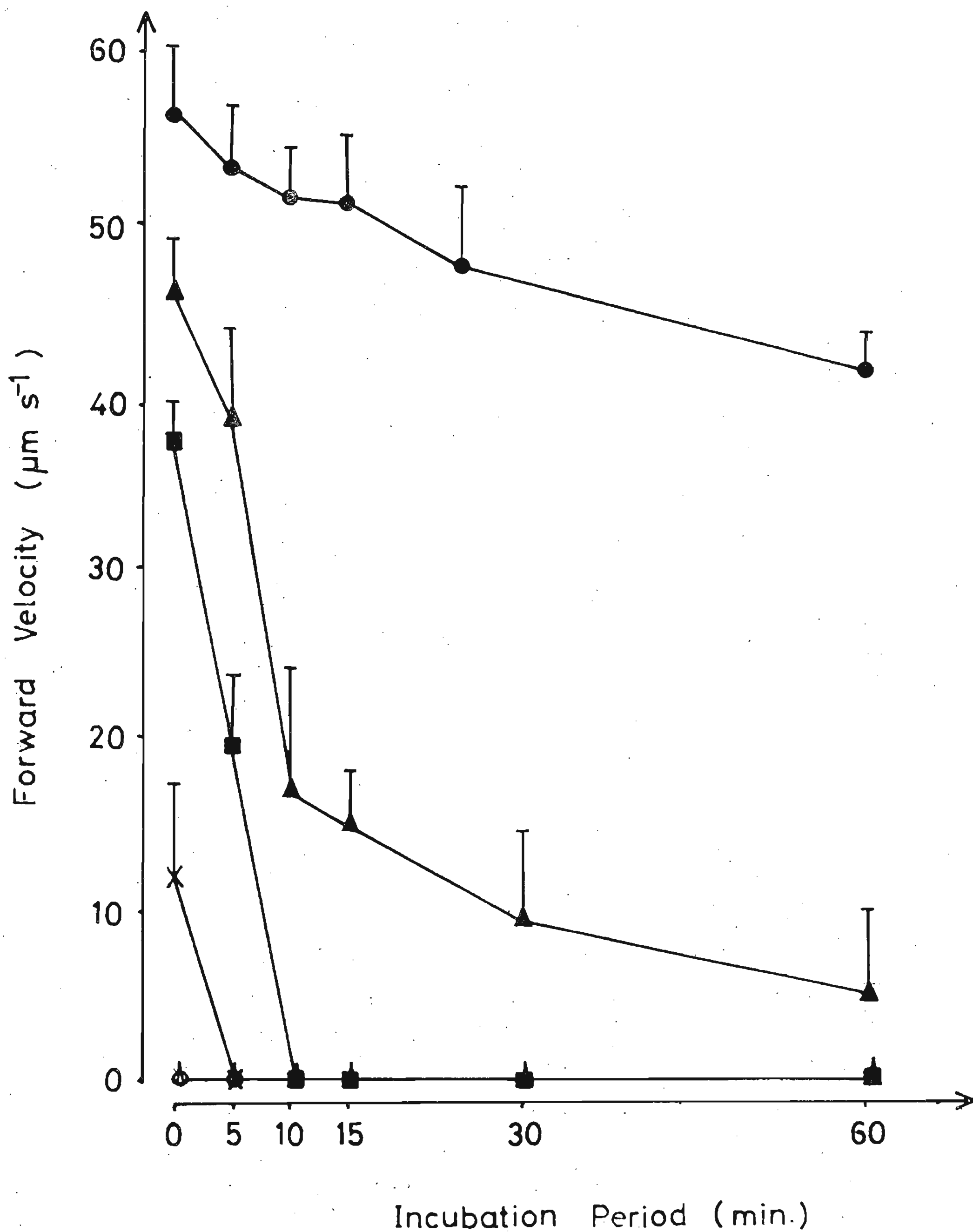

Figure 2 : Inhibition of average forward velocity of washed human spermatozoa by *Piper betele* leaf extract.

● = control ; ▲ = 0.25 mg ml^{-1} ; ■ = 0.5 mg ml^{-1} ;
x = 1.25 mg ml^{-1} ; ○ = 2.5 mg ml^{-1} ; Δ = 5.0 mg ml^{-1}.

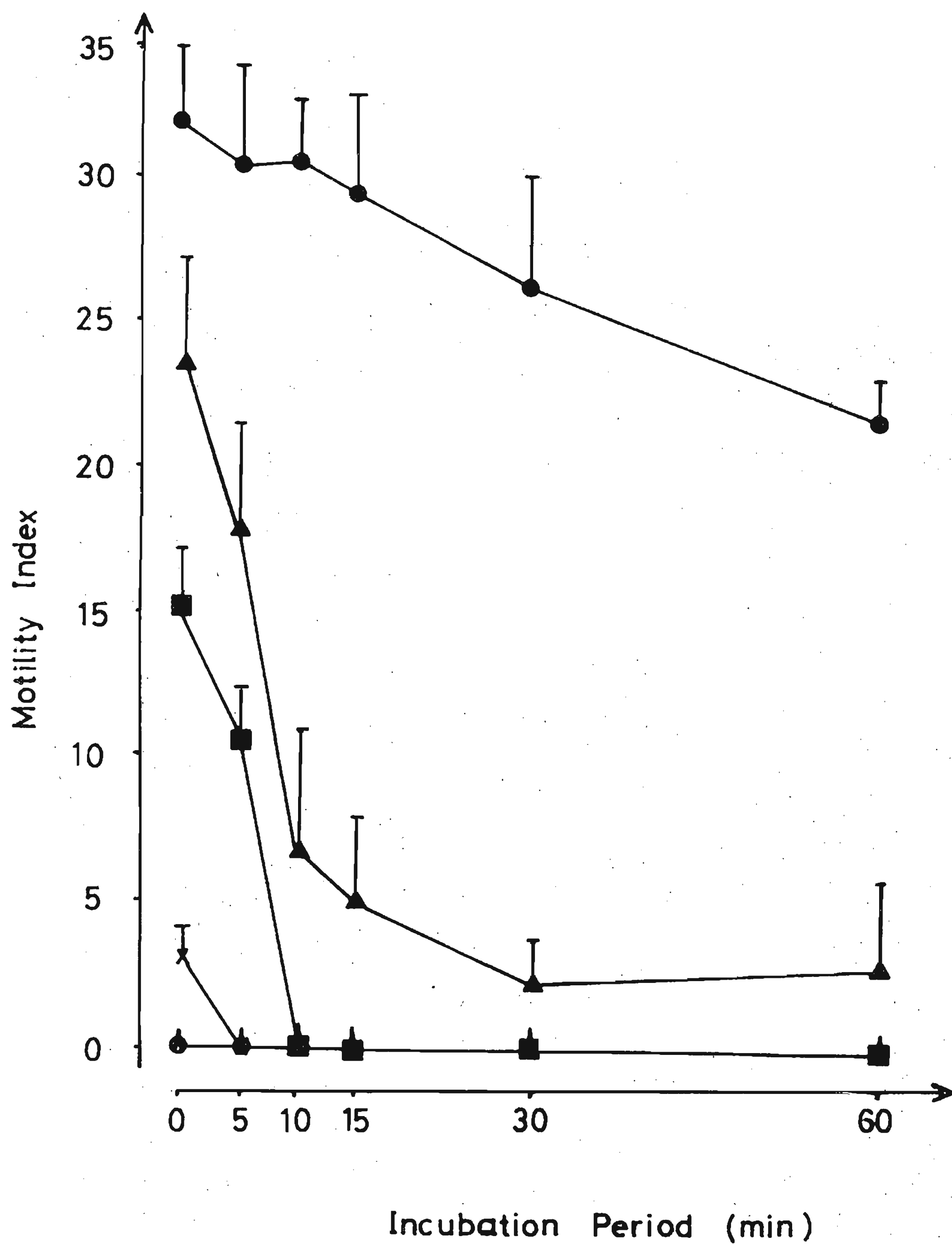

Figure 3 : Inhibition of motility index of washed human spermatozoa by *Piper betele* leaf extract.

● = control ; ▲ = 0.25 mg ml^{-1} ; ■ = 0.5 mg ml^{-1} ;
x = 1.25 mg ml^{-1} ; ○ = 2.5 mg ml^{-1} ; ∆ = 5.0 mg ml^{-1}.

4. Discussion

This study evaluates the antimotility properties of the crude extract of mature *Piper betele* leaves against washed human spermatozoa. The results demonstrate a concentration related impairment of percentage motility, average forward velocity and motility index.

The precise mechanism/s precipitating the antimotility action remains to be seen. Nevertheless, it is possible to rule out several potential mechanisms. Acidic conditions arrest spermatozoal motility,[12] but it is unlikely to be the causative factor to render spermatozoa immotile in this study since the extract had had a neutral pH in BWW medium. An agglutination of spermatozoa is a another mechanism known to inhibit motility[5] but such an agglutination was not evident. The presence of spermicidal properties in the extract also seems unlikely as the viability of the spermatozoa remained unaltered when assessed by the nigrosin–eosin stain technique[17]; spermicides cause permanent damage to the membranes resulting in death.[16] The fluidity of the plasma membrane too was unaltered as judged from the hypo–osmotic swelling test.[8] Under physiological conditions an intact and a fluid plasma membrane is certainly required for normal spermatozoal motility. Disruption of the activity of enzymes associated with motility can immobilize spermatozoa[12], but such a mechanism is usually time dependant and hence unlikely to be operative here.

The onset of the antimotility effects were almost instantaneous. Also the antimotility effects were irreversible.Coupled together,these observations suggest a direct action of a component/s in the extract on the plasma membrane of spermatozoa possibly by some form of strong binding.Binding domains have been demonstrated on the plasma membrane of spermatozoa[13] and certain drugs which are known to possess antimotility properties are claimed to bind firmly to the plasma membrane .[7,9] Such a binding may impair motility related functional competence of spermatozoa by effecting the fluxing of biologically important ions,[2] thus rendering them immotile. Spermtozoal motility is linked closely with fertility.[6] The fact that the extract abolished spermatozoal motility irreversibly at relatively low concentrations strongly suggest that potentially useful vaginal contraceptive agent/s may be isolated from *Piper betele* leaves. As such, activity directed fractionation of the extract should be undertaken. However, it is noteworthy that *in vitro* spermiostatic activity is always not predictive of similar *in vivo* action.

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