Male contraception is an interesting field of research throughout the world. Even World Health Organization (WHO) formed coalitions with Pakistan Government and international agencies to support research and activities in this field (1). A safe and effective method for contraception with the least side effects is a great need of today (2). Many products are available in market mainly composed of toxic materials. Repeated use cause inflammation and increases risk of sexual transmitted diseases (3). Several chemical and biological methods have been failed in this regard and are considered injurious for health (4).

Nowaday's, phytomedicines are matter of interest for scientist for male contraception because of less side effects and cost effectiveness (5). Spermicidal effects of plants like Gossypium herbaceum (6) Azadirachta indica (7), red algae G. acerosa (8) and Ruta graveolens (9) had been studied by researchers. Present study was designed to check the effect of Hippophae rhamnoides berries aqueous extract on human sperm motility by keeping in view the chemistry and folk use of plants as contraceptives.

MATERIAL AND METHODS

Collection, authentication and pulverization
Hippophae rhamnoides L. berries were collected from Cheema Laboratories, Civic Centre, Township Lahore and dried under the shade at room temperature for about 5 days. The dried berries were pulverized to a fine powder and stored in amber colored bottles.

Preparation of water extract
One hundred fifty grams of dried crushed berries were macerated in 1000 mL of distilled water, for 48 h at room temperature and in a dark place, and then filtered through a paper filter. The water from filtrate was removed in rotary vaccum evaporator. The reddish brown extract obtained was kept in clean jar at room temperature for further experiments. About 30 g of the extract was obtained.

Test for phenols
Crude extract (0.1 g) was dissolved in 2 mL of methanol and to this 2 mL of a 5% aqueous solution of ferric chloride was added. A development of purple coloration indicates the presence of phenols (10).

Collection of samples
Semen samples were obtained with masturba- tion, in diagnostic laboratory from ten patients reporting at Andrology Department, Fatima Memorial Hospital, Shadman, Lahore All patients were in between 35-45 year age. Written consent form was signed by each patient. Standardization of abstinence time i.e., 3-4 days was maintained. Samples were collected in wide mouth, transparent, sterile containers. Samples with azoospermia or severe oligozoospermia, sperm aggregation and sperm agglutination were excluded. The samples which had sperm motility between 50 and 70%, 20 × 10⁶ spermatozoa/mL, 2.1 mL /ejaculate, pH 7.5-7.9, and with minimum contamination of debris or
cells other than spermatozoa were used for the assay in accordance with the World Health Organization standard.

**Immobilization assay**

Sperm count motility was assessed microscopically as per the WHO manual (11). The samples were placed in an incubator at 37°C for 25-30 min. Subsequently, the samples were well mixed for 20 s at 37°C. Different amounts of the extract (2, 5, 10, 15 mg) were dissolved in 1 mL of physiological saline (0.9% NaCl) after filtration through a 0.2 µm filter; the filtrate was added to a semen sample in a 1 : 1 v/v ratio. In the control group, physiological saline (0.9% NaCl) was added to the same volume of semen. Samples were mixed for 10 s and tested for their effect on sperm motility. Ten µL of the mixture was immediately placed on a pre-warmed slide and 5 fields were viewed and at least 200 spermatozoa were counted in duplicate, i.e., 400 spermatozoa in total. At least five fields were assessed in each count. Within each field, all progressive spermatozoa (WHO class a) and slowly progressive spermatozoa (WHO class b) were counted first. All cells which were present in the field were counted. If the number of progressively motile spermatozoa were high in the field, a smaller part of the field was used. When all progressive spermatozoa were counted then non-progressive spermatozoa (WHO class c) and immotile spermatozoa (WHO class d) were counted in the same field. The four categories were expressed as percentages (rapid, slow, non-progressive, and immotile). Duplicate counting was made to detect and minimize random errors. Assessment of sperm motility was repeated on a second aliquot prepared in the same way. The averages for two counts were calculated and given as results. If the difference between the two motility counts was too large, two new assessments were made. The lowest dose causing 100% immobilization in aliquots was called the minimum effective dose (MED).

**Statistical data analysis**

All statistical calculations were performed with SPSS version 13 (Evaluation) for Windows. The results have been expressed as the mean ± S.E.%.

**RESULTS**

Purple coloration was evident with ferric chloride test, indicating the presence of phenolic compounds in the aqueous extract of *Hippophae rhamnoides* berries.

After preliminary screening of extracts (petroleum ether, chloroform, methanol), aqueous extract was selected for the study due to its good results and compatibility with semen samples. Different concentrations of aqueous extract were tested as described above and immediate immobilizing effects of the material were seen in a dose-dependent manner. The results showed that aqueous extract has antimotility activity at all concentrations with respect to control. It was also observed that sperm motility decreased with an increase in dose of extract. Maximum inhibition of sperm motility was observed at 15 mg/mL concentration, where all sperms become immotile immediately and was significantly different from control, 2 and 5 mg/mL concentrations (p = 0.000) but was insignificantly different from 10 mg/mL (p = 0.056). Fifteen mg/mL concentration was chosen as the minimum effective dose (MED) in other experiments. Sperm motility in 2 mg/mL was not significantly different (p = 0.719) from control group. The total motile sperm counts were significantly decreased in 5 mg/mL (p = 0.000) concentration, when compared with control and other concentrations (2, 10, 15 mg/mL). Sperm motility was also inhibited significantly in 10 mg/mL (p = 0.000) in comparison with control group, 2 and 5 mg/mL concentrations but the total motile sperm count in this concentration was insignificantly different from 15 mg/mL (p = 0.056) (Table 1).

It was also observed that pH gradually changed from alkaline to acidic. The lowest pH (6.5) was observed at concentration 15 mg/mL where sperm become totally immotile. Sperms were actively
motile in control and 2 mg/mL concentration group where pH was slightly alkaline (Table 1).

Eosin-nigrosin technique results had shown that the immobilized effect was with impairment in cell viability and losing membrane integrity. Treated cells were totally immotile at higher concentrations and there was a significant difference (p = 0.000) between viability of the two groups (control & MED) (Table 2)

**DISCUSSION**

In the present study, *in vitro* effects of aqueous extracts of *Hippophae rhamnoides* berries on human sperm motility were studied. These immobilizing effects were studied for the first time on the human sperm. The results (Tables 1, 2) showed that aqueous extract has maximum antimotility activity at higher concentration of 15 mg/mL; extract inhibits sperm motility immediately at this concentration. This might be due to strong interaction between sperm membrane and components of the extract which results in loss of membrane integrity and sperm viability. This was confirmed by eosin-nigrosin staining method (Table 2).

But still it is not known whether the immobilized effect was due to non-specific perturbation of plasma membrane (14) or due to decreased and disturbed functions of plasma membrane enzymes like Ca\(^{2+}\) ATPase (15) and Na-K-ATPase activity (16) or alternatively it could have resulted due to detergent action mediated by steroidal components i.e., sitosterol, isofucosterol, campsterol, stig mastanol, citrostadienol, av enasterol, cycloartenol, 24-ethyl-enecycloartanol and obtusifoliol which are reported to be present in berries (17), because Zia-Ul-Haq (18) reported that abridine (steroidal component present in *Abrus precatorius*) interact with sperm membrane by this mechanism.

Development of purple color with ferric chloride showed the presence of phenolic compounds in

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total motile sperm count % (Mean ± S.E.)</th>
<th>pH (p value)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1)</td>
<td>64.00 ± 2.66</td>
<td>7.9</td>
<td>(1-2) 0.719 (1-3) 0.000 (1-4) 0.000 (1-5) 0.000</td>
</tr>
<tr>
<td>2 mg/mL (2)</td>
<td>60.40 ± 2.38</td>
<td>7.9</td>
<td>(2-1) 0.719 (2-3) 0.000 (2-4) 0.000 (2-5) 0.000</td>
</tr>
<tr>
<td>5 mg/mL (3)</td>
<td>30.80 ± 2.61</td>
<td>7.5</td>
<td>(3-) 0.000 (3-2) 0.000 (3-4) 0.000 (3-5) 0.000</td>
</tr>
<tr>
<td>10 mg/mL (4)</td>
<td>8.00 ± 0.94</td>
<td>7</td>
<td>(4-1) 0.000 (4-2) 0.000 (4-3) 0.000 (4-5) 0.056</td>
</tr>
<tr>
<td>15 mg/mL (5)</td>
<td>0.000</td>
<td>6.5</td>
<td>(5-1) 0.000 (5-2) 0.000 (5-3) 0.000 (5-4) 0.056</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Percentage of viable sperm (mean ± S.E.)</th>
<th>15 mg/mL (MED)</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.03 ± 2.46</td>
<td>60.00 ± 2.66</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>
the extract. Phenolic and polyphenolic constituents of plants have a strong inhibitory effect on sperm motility with dose-response relationship (19).

A phenolic content ranges from 1.9-23.5 mg/g in Hippophae rhamnoides berries and leaves (20). Berries are rich source of polyphenolic compounds e.g., rutin, quercetin-3-O-galactoside, quercetin, myricetin, kaempferol and iso-rhamnetin. Phenolic acids include gallic, protocatechuic, p-coumaric, ferulic, p-hydroxybenzoic, catechin and ellagic acid.

Antimotility effect might be due to phenols and phenol containing compounds because these compounds stimulate lipid peroxidation and generate free oxygen species (ROS) (21). High levels of ROS in semen reduced sperm motility in vitro and cause damage to sperm nuclear DNA (22), sperm membranes and proteins due to interference and alterations in signal transduction mechanisms (23).

Phenols also cause disruption of ATP supply for sperm movement (24). Their activity was found to be dependent on the positions of hydroxyl groups and modulated by the positions of methyl groups on the benzene ring (21).

According to Carr et al. (25) low pH of the seminal fluid is sufficient to cause inhibition of sperm motility. This is in compliance with results because lowest pH was observed at higher concentration (15 mg/mL) that might be the reason of declining motility.

CONCLUSION

In conclusion, the results of the study strongly indicate that aqueous extract possesses contraceptive properties but further seminal and chemical investigations are required to find out the exact mechanism and site of action of aqueous extract on human spermatozoa.

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Received: 30. 10. 2015